PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Dan *et al.*	Group Art Unit: 1638
Serial No.: 10/715,910	Examiner: Kubelik, Anne R.
Filed: November 18, 2003	Atty. Dkt. No.: MONS:146US
FOR: USE OF LIPOIC ACID IN PLANT CULTURE MEDIA	Confirmation No.: 5658

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January 12, 2009 /Robert E. Hanson/
Date Robert E. Hanson

BRIEF ON APPEAL

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BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit this Appeal Brief. The date for filing this Brief is December 15, 2008, in view of the enclosed petition for extension of time and appropriate fee. The fee for filing this Brief is being concurrently filed. Should any additional fees become due under 37 C.F.R. §§ 1.16 to 1.21 for any reason relating to the enclosed materials, or should an overpayment be made, the Commissioner is authorized to deduct or credit said fees from or to Sonnenschein Nath & Rosenthal LLP Deposit Account No. 19-3140/MONS:146US.

I. REAL PARTY IN INTEREST

The Real Party in Interest is Monsanto Company, the parent company of assignee Monsanto Technology LLC.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-15 were filed with the application on November 18, 2003 and claims 5-15 were withdrawn from consideration by the Examiner as drawn to non-elected subject matter in the Office Action dated August 6, 2007. The claims were amended in the Response dated February 5, 2008. The amendment was entered by the Examiner and no subsequent amendments have been made. Claims 1-15 are currently pending. Claims 1-4 are under consideration and were finally rejected by the Examiner in the paper dated March 26, 2008 and are the subject of this appeal. A copy of the appealed claims as they currently stand is provided in Section VIII.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The subject matter defined in independent claim 1 comprises a method for introducing a nucleic acid sequence into the genome of a plant cell and regenerating a transformed plant therefrom, said method comprising culturing said plant cell on at least one plant transformation media, said at least one plant transformation media comprising an effective amount of lipoic acid or an analog thereof. (Specification, page 3, lines 12-21).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Were claims 1-4 properly rejected under 35 U.S.C. § 103(a) over Kulkarni *et al.* (U.S. Patent 6,365,407, filed March 2001) in view of Packer *et al.* ("Alpha-Lipoic Acid as a Biological Antioxidant"; 1995, *Free Rad. Biol. And Med.* 19:227-250)?

B. Were claims 1-4 properly rejected under 35 U.S.C. § 103(a) over Benson *et al.* (1997, *Phyton* 37(3):31-38) in view of Packer *et al.* ("Alpha-Lipoic Acid as a Biological Antioxidant"; 1995, *Free Rad. Biol. And Med.* 19:227-250)?

VII. ARGUMENT

A. The Claims Are Non-obvious Under 35 U.S.C. § 103(a) Over Kulkarni *et al.* In View Of Packer *et al.*

Claims 1-4 have been finally rejected as obvious under 35 U.S.C. § 103(a) over Kulkarni *et al.* (U.S. Patent 6,365,407, filed March 2001) in view of Packer *et al.* ("Alpha-Lipoic Acid as a Biological Antioxidant"; 1995, *Free Rad. Biol. And Med.* 19:227-250). In particular, the Examiner asserts that Kulkarni *et al.* disclose a method for culturing cells of a Himalayan Yew including a medium containing an antioxidant and that Packer *et al.* disclose lipoic acid as a biological antioxidant. The Examiner thus alleges that it would have been obvious to one skilled in the art as of the filing date to modify the method of culturing a plant cell on a medium comprising an antioxidant of Kulkarni *et al.* to use lipoic acid as the particular antioxidant in view of Packer *et al.* Appellants traverse for at least the reasons discussed below, and particularly based on the Examiner's reliance on impermissible hindsight, the fact that the claimed invention yields unexpected results, and because of the lack of any expectation in the art as of the filing date that the combination of cited art would succeed.

1. The Examiner Relies On Impermissible Hindsight Reasoning

The allegation that Kulkarni *et al.* and Packer *et al.* allegedly disclose the claimed method for transforming a plant cell using a transformation media comprising lipoic acid is based on impermissible hindsight reasoning and does not form the proper basis for a *prima facie* case of obviousness. In particular, while Packer *et al.* allegedly disclose lipoic acid as a biological antioxidant and Kulkarni *et al.* disclose culturing Himalayan Yew cells with an antioxidant for taxane production, nothing in the references or art general provides any suggestion or motivation to employ an antioxidant in connection with ***plant transformation***. Specifically, the claims are directed to a "method for ***introducing a nucleic acid sequence into the genome*** of a plant cell and ***regenerating a transformed plant therefrom***, said method comprising culturing said plant cell on at least one plant ***transformation media***, said at least one plant transformation media comprising an effective amount of lipoic acid or an analog thereof." (emphasis added).

No teaching has been shown by the Examiner in the cited art or alleged knowledge in the art generally, supporting a basis for employing an antioxidant in connection with a transformation media during the genetic transformation of a plant cell and regeneration of a transformed plant therefrom, let alone use of lipoic acid. The Examiner has not even shown that one of skill in the art would have had a desire to modify any existing transformation media in the prior art or methods for introducing a nucleic acid into a plant and regenerating a plant therefrom with the media. That is, the Examiner has not identified any shortcoming in the transformation media existing as of the filing date. Without such a showing, there would be no reason to make any modification to such media.

It would similarly not be "obvious to try" to modify any existing methods of genetically transforming and regenerating a transformed plant while employing a transformation media containing lipoic acid. The number of different variables that could be modified in a method of

plant transformation and transformation media used therein is nearly limitless. In the case of a transformation media alone, alterations could include adding any compound class not previously used in a transformation media, use of any different member of that given class, novel combinations of compounds, and different ratios of ingredients. Together, many thousands of different combinations could be made.

The Court in *KSR* noted that "[w]hen there is a ***design need or market pressure*** to solve a problem and there are a ***finite number of identified, predictable solutions***, a person of ordinary skill in the art has good reason to pursue the known options ***within his or her technical grasp***. If this leads to the ***anticipated success***, it is likely the product not of innovation but of ordinary skill and common sense." *KSR Int'l Co. v. Teleflex Inc.*, 550 U.S. 398 (U.S. 2007). In the current instance, the Examiner has not established any of these elements. Notably, no motivation to modify any existing transformation media has been provided or could be asserted, other than perhaps a generic desire to "make it better" that applies to any technology and is not a legitimate motivation at all given that: (1) absolutely anything would fall under such an approach, rendering it meaningless, and (2) the Examiner has not shown that the existing transformation media was considered as of the filing date to be in any way inadequate for existing needs, and thus capable of benefiting from modification. Finally, there are not a "finite" number of known solutions to modify existing plant transformation protocols, any such solutions are not predictable, and there would be no expectation of success in modifying the art to arrive at the claimed invention, as is established above.

In sum, the only disclosure of the use of an antioxidant for transformation of a plant or basis for believing that any benefit might be obtained therefrom is found in Appellants' own specification. The rejection is therefore based on hindsight reconstruction. A determination of

obviousness based on the appellants' specification is impermissible and does not support a *prima facie* case of obviousness (*see* MPEP §2142).

2. The Claimed Invention Yields Unexpected Results

In addition to the failure of the cited references to teach or suggest the claimed invention, the invention yields surprising and unexpected results affirmatively demonstrating the non-obviousness of the claimed invention. Specifically, the working examples of the specification demonstrate that the claimed invention results in an increase in transgenic plant production, heterologous gene expression, transformation efficiency and a decrease in tissue browning. This would in no way be predicted by one of ordinary skill in the art as of the filing date.

For instance, the working examples of the current application demonstrate both an increase in the percentage of transgenic plants produced per explant by 2.7 to 4.3 fold, as well as an increase in transient expression of a heterologously introduced marker gene for a cultivar of tomato species, *Lycopersicon esculentum* when lipoic acid was used. In particular, the impact of lipoic acid on the percentage of transgenic plants produced per explant is shown in Table 3, reproduced below. Treatment with 10uM lipoic acid increased the percentage of transgenic plants produced per explant from 40.1% to 178.7%.

TABLE 3

Effect of lipoic acid on cell browning (5 days after selection), transient GUS expression (5 days after selection), transformation efficiency (TE) (percentage of transgenic events produced per explant), and stable transformation efficiency (percentage of transgenic plants produced per explant).

Lipoic Acid conc. (μM)	% low browning	% high transient expression	TE	Efficiency
0	36.1	30	27.9	41.4
5	65.1	42.9	78.3	155.1
10	66.1	60.9	94.1	178.7
50	65.6	56.5	76.4	144.1
100	54	34.8	49.7	112.1

(Specification, page 33)

Similarly, the impact of lipoic acid on expression of the marker gene beta-glucuronidase (GUS) is shown in Table 2, reproduced below. Treatments of lipoic acid at concentrations of 10 and 50uM increased the percentage of explants having high transient expression up to 60.9%. Additionally, when lipoic acid was used at concentrations of 10, 50 and 100uM, 100% of explants transiently expressed the GUS gene.

TABLE 2

Effect of lipoic acid on transient GUS expression after 5 days on selection media.

Lipoic Acid conc. (μM)	% High transient expression	% Low transient expression	% No transient expression
0	30	50	20
5	42.9	47.6	9.5
10	60.9	39.1	0
50	56.5	43.5	0
100	34.8	65.2	0

(Specification, page 32)

The effect of lipoic acid on browning of tissue was also assessed. Tissue browning was measured in and around a poked region on cotyledon explants. All treatments of lipoic acid at concentrations of 5, 10, 50, and 100uM had a 1.5 to 1.8 fold increase in the number of explants scored as low tissue-browning severity, respectively, compared to the treatment without lipoic acid. See Table 1, reproduced below.

TABLE 1

Effect of lipoic acid on tissue browning after 5 days on selection media.

Lipoic Acid Conc. (μM)	% low browning	% high browning
0	36.1	63.9
5	65.1	34.9
10	66.1	33.9
50	65.6	34.4
100	54	46

(Specification, page 31)

Similar effects were seen in lipoic acid treatments of a potato cultivar, Ranger Russet. Results in Table 7, reproduced below, depict the 6 fold increase in transformation efficiency, to 19% and 18% at 8uM and 9uM concentrations, respectively. Lipoic acid also reduced the percentage of escapes (non-transgenic shoots) from 50% in the control to 16% and 21% of 8uM and 9uM treatments, respectively.

TABLE 7

Effect of lipoic acid on transformation in potato (TE = transformation efficiency).

lipoic acid µM	#explants	shoots	#rooted	% TE	% escape
6	400	27	18	4.5	33.0
7	400	25	16	4.0	36.0
8	400	89	75	19.0	16.0
9	400	89	70	18.0	21.0
control	400	20	10	3.0	50.0

(Specification, page 39)

Positive results are also shown by the working examples in wheat (Example 3), soybean (Example 4) and cotton (Example 5). For instance, Table 9 of Example 3 which, is reproduced below, shows that when 50 µM of lipoic acid was used in the delay, selection, and regeneration media, the percentage of responding wheat calli was increased from 32.5% to 47.9% and increases observed in efficiency from 3.0% to 5.1%. When different concentrations were tried in the delay, selection and first regeneration media (Table 10, also reproduced below), 25 µM in the delay medium, 50 µM in the selection medium, and 50 µM in the first regeneration medium also were effective, giving 5.4% transformation efficiency compared with 2.9% in the control. The results for 50 µM in each medium corresponds with the previous experiment, giving 5.4% transformation efficiency.

Table 9: Different concentrations of lipoic acid in delay, selection and first regeneration media.

Concentration (µM)	Explants	Responding calli (%)	Efficiency (%)
0	470	32.8	3.0
5	472	33.1	3.8
10	486	38.8	3.2
30	486	38.6	3.0
50	482	47.9	5.1
100	464	28.4	2.2

(Specification, page 43)

Positive results were also obtained with different lipoic acid concentrations of selection and regeneration in wheat (Table 10, below).

Table 10. Effect of lipoic acid at different stages of selection and regeneration.

Delay-selection-first regeneration	Explants	% responding calli	% efficiency
0-0-0	582	40.8	2.9
25-25-50 (µM)	599	41.7	3.0
25-50-50 (µM)	560	50.5	5.4
50-50-50 (µM)	570	49.6	4.9

(Specification, page 43)

Similarly, in the case of soybean transformation, while low lipoic acid concentrations were not effective, "lipoic acid concentrations of 250 µM to 1500 µM provided increased transformation efficiency and increased shoot and rooted plant production. A lipoic acid concentration of 250 µM increased transformation frequency about ninefold."

Table 11. Effect of lipoic acid in soybean with effects of dissolving in ethanol or potassium hydroxide.

Treatment	Total Explants	Total Shoots	Shooting Frequency	Rooted Plants	Transformation Efficiency
Control, 0 uM	472	25	5%	4	0.8%
0.05% ETOH	478	34	7%	10	2.1%
250uM LA	486	69	14%	7	1.4%
250uM LA, KOH	505	63	12%	13	2.6%
0.10% ETOH	556	44	8%	9	1.6%
500uM LA	531	64	12%	9	1.7%
500uM LA, KOH	462	44	10%	7	1.5%
0.15% ETOH	551	33	6%	3	0.5%
750uM LA	535	62	12%	9	1.7%
750uM LA, KOH	454	56	12%	7	1.5%
0.20% ETOH	485	30	6%	6	1.2%
1000uM LA	530	52	10%	12	2.3%
1000uM LA, KOH	471	40	8%	6	1.3%
0.30% ETOH	483	30	6%	3	0.6%
1500uM LA	538	53	10%	13	2.4%
1500uM LA, KOH	498	57	11%	10	2.0%

Table 12. Effect of lower levels of lipoic acid on soybean.

Treatment	Total Explants	Total Shoots	Shooting Frequency	Total Rooted Plants	Transformation Efficiency
Control, 0 uM	490	37	8%	3	0.6%
5 uM LA	244	18	7%	3	1.2%
5 uM LA, KOH	248	13	5%	2	0.8%
10 uM LA	247	14	6%	0	0.0%
10 uM LA, KOH	240	13	5%	0	0.0%
50 uM LA	242	22	9%	1	0.4%
50 uM LA, KOH	246	17	7%	1	0.4%
100 uM LA	396	39	10%	3	0.8%
100 uM LA, KOH	246	18	7%	2	0.8%
250 uM LA	246	32	13%	9	3.7%
500 uM LA*	171	19	11%	5	2.9%

(Specification, pages 45-46)

Finally, in the case of cotton, Example 5 states that "[a]s shown in Table 13, the inclusion of lipoic acid at a concentration of 50 μM or 100 μM in the selection stage of cotton transformation and regeneration increased the frequency of embryogenic callus formation from 41.4% in the control to 61.2% and 56.6%, respectively." The corresponding table is reproduced below.

Table 13: Effect of lipoic acid for 8 weeks in UMSEL media (average from 3 experiments).

Lipoic acid (μM)	Putative embryogenic calli	Frequency (%)
0	121	41.4
5	132	49.4
10	88	31.4
50	120	61.2
100	107	56.6

(Specification, page 50)

The foregoing results represent a significant advance that would in no way have been expected based on the knowledge in the art. In the present case, the results obtained by the invention are anything but predictable and the Examiner's allegations that the above results are not unexpected or persuasive are unfounded. Nothing in the art suggests a use for lipoic acid in transformation at all, let alone significant benefits, thus the above results are unexpected. The Examiner incorrectly asserts that the Appellants must show that lipoic acid is better than other antioxidants as, again, nothing in the art suggests any benefit or even a basis for making it "obvious to try" to use an antioxidant in the context of plant transformation, thus the results establish the non-obviousness of the claimed invention.

3. The Cited References Provide No Expectation Of Success

One of skill in the art would have further had no expectation of success in arriving at the invention in view of the combination of Kulkarni *et al.* and Packer *et al.* In particular, the claimed invention relates to a method for introducing a nucleic acid sequence into the genome of a plant cell and regenerating a transformed plant therefrom comprising use of a plant transformation media with an effective amount of lipoic acid. Kulkarni *et al.* allegedly disclose culturing cells of the Himalayan Yew on a medium comprising "a number of antioxidants" in

order to promote taxane production. The cited art fails to teach or suggest the use of ***any*** antioxidant in connection with ***a transformation media***, let alone lipoic acid, as is claimed.

In particular, Kulkarni *et al.* mentions that, in culturing a Himalayan Yew cell, the antioxidants may function only to ***aid in taxane production***. In fact, even the working examples of Kulkarni *et al.* indicate that an antioxidant is optional, as the studies were carried out "***with or without antioxidants***." See Kulkarni *et al.* at column 5, lines 11-14. Packer *et al.* similarly relate to culturing mammalian cells and do not to teach any connection between lipoic acid and plant cells or plant genetic transformation. One of skill in the art would therefore not expect the use of lipoic acid, let alone any antioxidant, to yield successful results in the context of the current invention. .

In sum, the Examiner's reliance on impermissible hindsight, the presented unexpected results of the invention and the lack of any expectation of success in view of the cited references more than establish the non-obviousness of the clamed invention. Reversal of the rejection is thus respectfully requested.

B. The Claims Are Non-obvious Under 35 U.S.C. § 103(a) Over Benson *et al.* In View Of Packer *et al.*

Claims 1-4 have been finally rejected as obvious under 35 U.S.C. § 103(a) over Benson *et al.* (1997, *Phyton* 37(3):31-38) in view of Packer *et al.* ("Alpha-Lipoic Acid as a Biological Antioxidant"; 1995, *Free Rad. Biol. And Med.* 19:227-250). In particular, the Examiner asserts that Benson *et al.* disclose a method comprising culturing a plant cell on a media, and that plant cell culture is affected by free radicals, while Packer *et al.* teach that lipoic acid is a biological antioxidant. It is further asserted that it would have been obvious to one of ordinary skill in the art to modify the method of Benson *et al.* to use lipoic acid, as described by Packer *et al.,* as an

antioxidant. Appellants respectfully request reversal of the rejection, as the Examiner has relied on hindsight reasoning, the claimed invention provides unexpected results, and there would be no expectation to one of ordinary skill in the art as of the filing date that the combination of the cited references would succeed in the context of the claimed invention, as discussed below.

1. The Cited References Provide No Expectation Of Success

The cited references, in combination or alone, fail to provide any expectation of success, and if anything, teach away from the claimed invention. In particular, the claimed invention is drawn to "[a] method for introducing a nucleic acid sequence into the genome of a plant cell and regenerating a transformed plant therefrom, said method comprising culturing said plant cell on at least one plant transformation media... comprising an effective amount of lipoic acid or an analog thereof." As explained in detail above, Packer *et al.* does not relate to plant transformation, and rather relates to culturing of mammalian cells. Additionally, while Benson *et al.* allegedly teach that plant cell culture is affected by free radicals, the reference itself states that "at present there exists no direct evidence to implicate free radicals, activated oxygen species and/or their reaction products as causal agents in either genetic or epigenetic instability in plant cultures." (Benson *et al.*, page 35). The authors also repeatedly warn that oxidative processes may have a positive and direct role in *in vitro* development, and caution against considering only the negative aspects of free radicals. For instance, it is stated that "there now exists considerable evidence to support the view that ***free radicals are a component of in vitro plant development***" and that "metabolic and developmental pathways have ***a dependency on activated oxygen*** (for example, those involving lipoxygenase)." Benson *et al.,* pages 32 and 35, respectively; emphasis added. Similarly it is stated on page 33 that:

> [T]he lipid peroxidation product, jamonic acid ***can stimulate in vitro plant morphogenesis*** (Ravnikar & Gogala 1990). Similarly, Earnshaw & Johnson 1985 correlated glutathione status with morphogenetic competence in carrot suspension

> cultures. GSH levels were higher in proliferating cultures compared to differentiating cultures, and they concluded that ***development occurs in a more oxidising environment***.

(emphasis added) The authors then state that "[i]t is thus essential to consider both positive and negative aspects of *in vitro* oxidative metabolism in plants" and that "sole consideration of the negative aspects of free radical activity risks over simplification." (Benson *et al.*, page 33)

Benson *et al.* therefore clearly discloses that free radicals may have a beneficial impact on cell cultures. A person of skill in the art would thus not interpret the reference to teach or suggest the use of antioxidants in tissue culture in general, as the authors themselves underscore the lack of understanding of antioxidants and the role of free radicals in plant culture and development. This clearly demonstrates the lack of any reasonable expectation of success one of the skill in the art would have had in using lipoic acid in a method for introducing a nucleic acid sequence into the genome of a plant cell and regenerating a transformed plant therefrom, not to mention the lack of any teaching with respect to plant transformation, as recited in the claims.

The Examiner, however, argues on page 4 of the final Office Action that the reference provides strong directive to use antioxidants in plant culture, citing the authors' statement that "parallel analyses of pro- and antioxidants must be performed." (Benson *et al.*, page 36). However, when the full corresponding sentence is read as follows: "[m]eaningful investigations can only be carried out on well characterized cultures and parallel analyses of pro- and antioxidants must be performed." (*Id.*), the statement is actually cautionary of the use of antioxidants without further study of ***pro- and*** antioxidants. This demonstrates that, at most, antioxidants might be beneficial or might be detrimental and it does not teach the use of antioxidants in plant transformation media, let alone lipoic acid in particular.

The Examiner further argues on page 5 of the final Office Action that Benson *et al.* suggests testing the effects of antioxidants on plant tissue culture, citing the paragraph spanning page 36-37, reproduced below.

> Substantial correlative evidence supports the premise that free radical mechanisms have a role in in vitro plant development. Future studies must utilise stringent experimental strategies and employ more discerning methods for the analysis of free radical products. Meaningful investigations can only be carried out on well characterised cultures and parallel analyses of pro- and antioxidants must be performed. Oxidative status and morphogenetic capacity may be interrelated and both may be influenced by subculture cycle, culture age and genotype (Fig.1). We strongly caution the use of tissue cultures as "models" for the study of stress responses without prior knowledge of the culture's developmental history. Habituation, culture age and competence greatly influence the oxidative status of cultures and may confound experimental interpretations. Finally, the application of transformation technologies offers immense potential for the study of antioxidant systems in plants (BADIANI & al. 1996). However, if transgenic in vitro systems are to be utilised it may be useful to be aware of the interactions between culture competence, age and oxidative status.

As shown above, in the same paragraph as that cited by the Examiner, the authors actually ***caution against the use of antioxidants in plant culture media*** without further experimentation, specifically stating that there is "substantial correlative evidence" that ***free radicals play a role in plant development***. If anything, the paragraph referred to by the Examiner teaches away from the invention, as reducing free radicals would presumably inhibit plant development and thus not be desirable as a culture component.

The combination of Benson *et al.* and Packer *et al.* therefore provides no expectation that addition of lipoic acid as an antioxidant, or addition of ***any*** antioxidant, to a plant transformation media or use in a method to introduce a nucleic acid sequence into the genome of a plant cell and regenerate the transformed plant would yield successful results. In fact, in view of the disclosure

that free radicals may play a positive role in development, Benson *et al.* teaches away from the addition of an antioxidant to plant culture media in general.

2. The Claimed Invention Yields Unexpected Results

Appellants further note that the current invention provides surprising and unexpected results that in no way could be predicted by the art, thus demonstrating the non-obviousness of the invention. As noted above, the working examples illustrate an increase in the percentage of transgenic plants produced per explant by 2.7 to 4.3 fold, as well as an increase in transient expression of a heterologously introduced marker gene when lipoic acid was used. The impact of lipoic acid on the percentage of transgenic plants produced per explant is shown in Table 3, reproduced above. Treatment with 10uM lipoic acid increased the percentage of transgenic plants produced per explant from 40.1% to 178.7%. Similarly, the impact of lipoic acid on expression of the marker gene beta-glucuronidase (GUS) is shown in Table 2, reproduced above. Treatments of lipoic acid at concentrations of 10 and 50uM increased the percentage of explants having high transient expression from 10% to 60.9%. A significant decrease in tissue browning was also observed during tissue culture as demonstrated in Table 1, reproduced above. Similar unexpected results were obtained for lipoic acid treatments on potato plants, Table 7, reproduced above. In wheat, lipoic acid treatments were identified that increased the percentage of responding calli from 32.5% to 47.9%, and increased observed efficiency from 3.0% to 5.1%. Table 10 reports that, using different combinations of media with lipoic acid, 5.4% transformation efficiency was obtained compared with 2.9% in the control.

In soybeans, lipoic acid concentrations of 250 μM to 1500 μM provided increased transformation efficiency and increased shoot and rooted plant production, with 250 μM of lipoic acid increasing transformation frequency about ninefold (Tables 11 and 12). Finally, in cotton, lipoic acid at a concentration of 50 μM or 100 μM in the selection stage of cotton transformation

and regeneration increased the frequency of embryogenic callus formation from 41.4% in the control to 61.2% and 56.6%, respectively (Table 13).

In the present case the yielded results are anything but expected or predictable based on the state of the art. For example, Packer *et al.* relates to lipoic acid in relation to various mammalian systems and Benson *et al.* is simply asserted to teach that tissue culture is affected by free radicals, yet, the authors also teach away from the use of antioxidants in tissue culture by noting that "free radicals are a component of *in vitro* plant development." One of skill in the art would thus not interpret these references as predicting any positive effect on transgenic plant production, plant cell transformation and gene expression when using a tissue culture containing any antioxidant, let alone lipoic acid used in a method to introduce a nucleic acid sequence into the genome of a plant cell and regenerate a transformed plant therefrom. Thus, the results disclosed in the working examples of the present application are surprising and unexpected, and therefore demonstrate the non-obviousness of the claimed invention.

3. The Examiner Relies On Impermissible Hindsight Reasoning

The Examiner asserted on pages 3 and 4 of the Office Action dated August 6, 2007 that one of skill in the art would have been motivated to combine the cited art and introduce a nucleic acid sequence into the genome of a plant cell and regenerate a transformed plant therefrom using a transformation media containing lipoic acid. This amounts to pure speculation and impermissible hindsight reconstruction. Nothing in the cited references or art generally would lead one of skill in the art to arrive at the claimed invention.

The authors of Benson *et al.* themselves teach the ***positive effects of free radicals on in vitro plant development*** and Packer *et al.* discuss culturing of mammalian cells generally and not plant transformation. Thus, the only teaching or suggestion of using lipoic acid, or any antioxidant for matter, in a plant transformation media is found in the Appellants' own

specification, together with unexpected results including an increase in the percentage of transgenic plants produced per explant by a 2.7 to 4.3 fold, an increase in transient expression of a marker gene, and reduction of browning during tissue culture. A determination of obviousness based on the appellants' specification is impermissible and does not constitute a *prima facie* case of obviousness. MPEP §2142.

In view of the above demonstration that the cited art does not provide reasonable expectation of success and, if anything teaches away from the invention, the unexpected and surprising results provided by the invention and the Examiner's reliance on impermissible hindsight, Appellants therefore respectfully request reversal of the obviousness rejection.

CONCLUSION

It is respectfully submitted, in light of the above, that none of the claims are properly rejected. Therefore, Appellants request that the Board reverse the pending grounds for rejection.

Respectfully submitted,

/Robert E. Hanson/

Robert E. Hanson
Reg. No. 42,628
Attorney for Appellants

SONNENSCHEIN NATH & ROSENTHAL LLP
2000 McKinney, Suite 1900
Dallas, TX 75201
(214) 259-0900

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VIII. CLAIMS APPENDIX

APPEALED CLAIMS:

1. A method for introducing a nucleic acid sequence into the genome of a plant cell and regenerating a transformed plant therefrom, said method comprising culturing said plant cell on at least one plant transformation media, said at least one plant transformation media comprising an effective amount of lipoic acid or an analog thereof.

2. The method of claim 1 wherein the amount of lipoic acid or an analog thereof in said plant transformation media is between about 2 μM and about 2000 μM.

3. The method of claim 1 wherein the amount of lipoic acid or an analog thereof in said plant transformation media is between about 5 μM and about 1500 μM.

4. The method of claim 1 wherein the amount of lipoic acid or an analog thereof in said plant transformation media is between about 5 μM and about 100 μM.

IX. EVIDENCE APPENDIX

Exhibit A: Kulkarni *et al.* (U.S. Patent 6,365,407, filed March 2001).

Exhibit B: Packer *et al.* ("Alpha-Lipoic Acid as a Biological Antioxidant"; 1995, *Free Rad. Biol. And Med.* 19:227-250).

Exhibit C: Benson *et al.* ("Free Radical Processes in Plant Tissue Cultures: Implications for Plant Biotechnology Programmes"; 1997, *Phyton* 37(3):31-38).

EXHIBIT A

EXHIBIT B

Pergamon

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Review Article

ALPHA-LIPOIC ACID AS A BIOLOGICAL ANTIOXIDANT

LESTER PACKER,* ERIC H. WITT,* and HANS JÜRGEN TRITSCHLER†

*Membrane Bioenergetics Group and Department of Molecular & Cell Biology, University of California, Berkeley, CA, USA; and †ASTA Medica, Frankfurt Am Main, Germany

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Abstract—α-Lipoic acid, which plays an essential role in mitochondrial dehydrogenase reactions, has recently gained considerable attention as an antioxidant. Lipoate, or its reduced form, dihydrolipoate, reacts with reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen. It also protects membranes by interacting with vitamin C and glutathione, which may in turn recycle vitamin E. In addition to its antioxidant activities, dihydrolipoate may exert prooxidant actions through reduction of iron. α-Lipoic acid administration has been shown to be beneficial in a number of oxidative stress models such as ischemia–reperfusion injury, diabetes (both α-lipoic acid and dihydrolipoic acid exhibit hydrophobic binding to proteins such as albumin, which can prevent glycation reactions), cataract formation, HIV activation, neurodegeneration, and radiation injury. Furthermore, lipoate can function as a redox regulator of proteins such as myoglobin, prolactin, thioredoxin and NF-κB transcription factor. We review the properties of lipoate in terms of (1) reactions with reactive oxygen species; (2) interactions with other antioxidants; (3) beneficial effects in oxidative stress models or clinical conditions.

Keywords—Antioxidant, Dihydrolipoate, Dihydrolipoic acid, α-Lipoate, α-Lipoic acid, Oxidative stress, Redox regulation, Review, Thioctic acid, Free radicals

INTRODUCTION

The metabolic role of α-lipoic acid (Fig. 1) has been known for decades. It was first isolated by Reed and coworkers as an acetate replacing factor[1,2] and is insoluble in water, but soluble in organic solvents. It is known by a variety of names, including thioctic acid; 1, 2-dithiolane-3pentanoic acid; 1, 2-dithiolane-3 valeric acid; and 6,8-thioctic acid. As lipoamide, it functions as a cofactor in the multienzyme complexes that catalyze the oxidative decarboxylation of α-keto acids such as pyruvate, α-ketoglutarate, and branched chain α-keto acids.[3] α-Lipoic acid was tentatively classified as a vitamin after its isolation,[1,4] but it was later found to be synthesized by animals and humans[5]; however, the complete enzyme pathway that is responsible for the de novo synthesis has not yet been elucidated. Several studies indicate that octanoate serves as the immediate precursor for the 8-carbon fatty acid chain, and cysteine appears to be the source of sulfur.[6]

More recently, a great deal of attention has been given to possible antioxidant functions for α-lipoic acid and its reduced form, dihydrolipoic acid (DHLA, Fig. 1). In this review we are concerned with the antioxidant properties of these compounds and the preventive and therapeutic implications and applications that arise from this new view of an old cofactor.

Antioxidant criteria

Many criteria must be considered when evaluating the antioxidant potential of a compound. Some of these concern chemical and biochemical aspects:

Dr. Lester Packer is a professor in the Department of Molecular and Cell Biology at the University of California at Berkeley. He received a Ph.D. in Microbiology and Biochemistry from Yale University in 1956. At Berkeley he has been Professor of Physiology since 1961, Director of the Membrane Bioenergetics Group at the University of California at Berkeley, and a Senior Scientist at the Lawrence Berkeley Laboratory since 1972. His research, in the area of biological oxidation and bioenergetics has emphasized studies on the role of oxidants and antioxidants in biological systems.

He is one of the world's leading researchers on vitamin E and biological antioxidants. His recent work has elucidated new areas in the biochemistry of vitamin E—the vitamin E cycle. This is relevent to the understanding of new enzymic reactions of vitamin E, the vitamin E radical, and the biological consequences of the action of vitamin E. Recent work concerns the prevention of oxidatively induced injury in biological systems by antioxidants and how antioxidants such as vitamin E and lipoic acid affect gene expression and cell regulation. These investigations are helping to develop a new broader understanding of biological antioxidant defense mechanisms.

Dr. Packer has edited more than 50 books, authored over 500 articles, is a member of 8 professional societies and 3 editorial boards of scientific journals, and has organized numerous conferences in the area of his research interests. Currently he is President of The Oxygen Club of California, President Elect of International Society for Free Radical Research, and Vice President of UNESCO's Global Network on Molecular and Cell Biology.

Address correspondence to: Lester Packer, 251 Life Sciences Addition, University of California, Berkeley, CA 94720, USA.

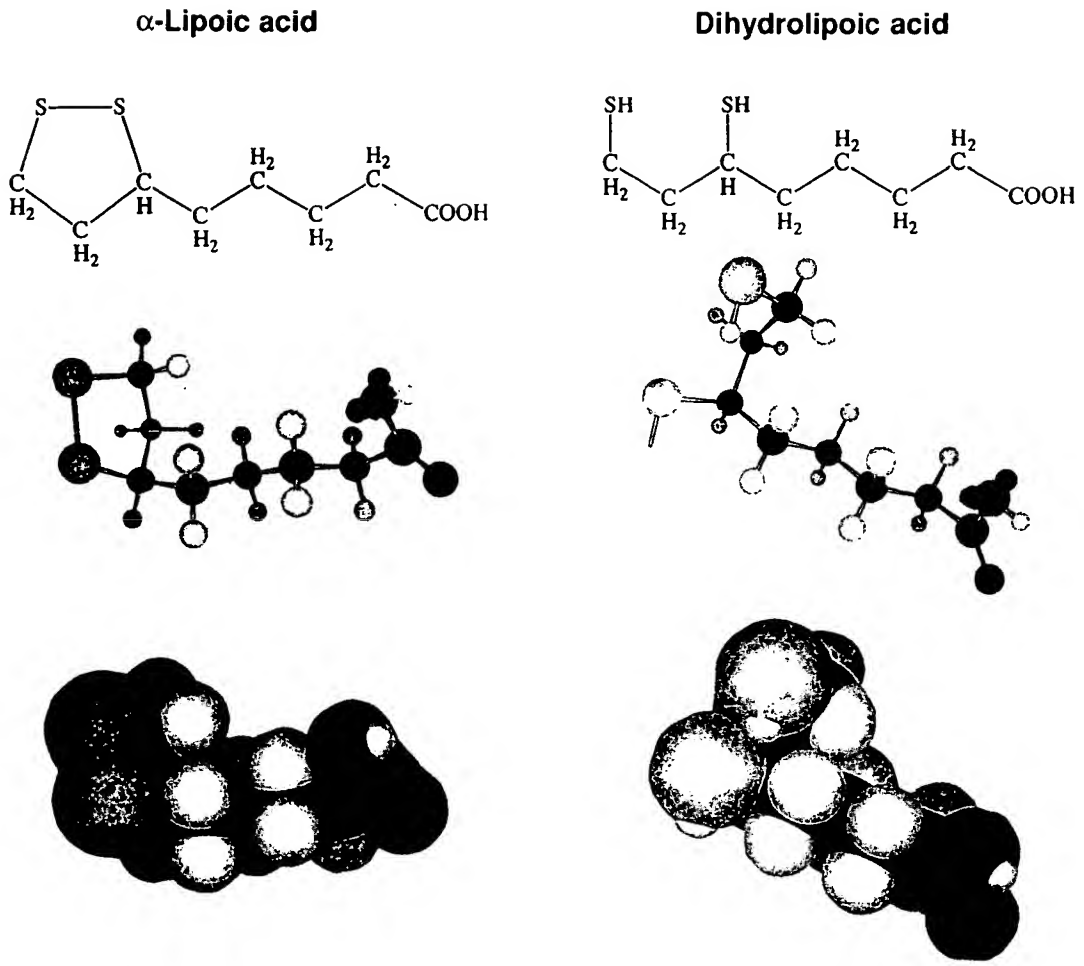

Fig. 1. The structures of α-lipoic acid and dihydrolipoic acid, shown as chemical structures (top), ball-and-stick models (middle), and space-filling models (bottom). The naturally occurring R-enantiomers are shown. Synthetic α-lipoic acid is a racemic mixture of the R- and the S-enantiomer.

- Specificity of free radical quenching
- Metal chelating activity
- Interaction with other antioxidants
- Effects on gene expression

Other criteria are important when considering preventive or therapeutic applications:

- Absorption and bioavailability
- Concentration in tissues, cells, and extracellular fluid
- Location (in aqueous or membrane domains or in both?)

A substance need not excel in meeting all these criteria to be considered a good antioxidant. For example, vitamin E acts only in the membrane or lipid domains, its dominant action is to quench lipid peroxyl radicals, and it has little or no activity against radicals in the aqueous phase, yet it is considered one of the central antioxidants of the body. Epidemiological studies are confirming its role in the prevention of numerous oxidant-related diseases, such as heart disease.[7,8]

An "ideal" antioxidant would fulfill all of the above criteria. The α-lipoic acid/dihydrolipoic acid redox couple approaches the ideal; it has been called "a universal antioxidant."[9] α-Lipoic acid is readily absorbed from the diet. It is probably rapidly converted to DHLA in many tissues, as recent advances in assay technique have made evident.[10,11] One or both of the components of the redox couple effectively quench a number of free radicals in both lipid and aqueous domains. Both DHLA[21,32,33] and α-lipoic acid[13,19,23,24] have metal-chelating activity. DHLA acts synergistically with other antioxidants, indicating that it is capable of regenerating other antioxidants from their radical or inactive forms. Finally, there is evidence that they may have effects on regulatory proteins and on genes involved in normal growth and metabolism.

Because of these antioxidant attributes, a number of experimental and clinical studies have been carried

out which show α-lipoic acid to be useful or potentially useful as a therapeutic agent in such conditions as diabetes, ischemia-reperfusion injury, heavy-metal poisoning, radiation damage, neurodegeneration, and HIV infection.

ANTIOXIDANT ACTIONS

An antioxidant function for α-lipoic acid was suggested as early as 1959 by Rosenberg and Culik,[4] who observed that administration of α-lipoic acid prevented scurvy symptoms in vitamin C-deficient guinea pigs as well as preventing symptoms of vitamin E deficiency in rats fed a diet lacking α-tocopherol. It has only been recently, however, that the specific effects of α-lipoic acid and DHLA in free radical quenching, metal chelation, antioxidant recycling, and gene expression have been investigated.

Reactive oxygen species quenching and metal chelation

Lipoic acid. There is general agreement about the antioxidant properties of α-lipoic acid. It scavenges hydroxyl radicals, hypochlorous acid, and singlet oxygen. It does not appear to scavenge hydrogen peroxide or superoxide radical and probably does not scavenge peroxyl radicals (Table 1). It may chelate transition metals.

Two studies indicate that α-lipoic acid is a potent hydroxyl radical scavenger. In one,[12] hydroxyl radical was generated by 2 mM H_2O_2 + 0.2 mM $FeSO_4$. The radical was detected by electron spin resonance (ESR) using the spin-trapping agent 5,5-Dimethylpyrroline-N-oxide (DMPO). 1 mM α-lipoic acid completely eliminated the DMPO-OH adduct signal. Another study,[13] using a similar hydroxyl radical-generating system (2.8 mM H_2O_2, 0.05 mM $FeCl_3$, 0.1 mM EDTA, and 0.1 mM ascorbate) but a different assay for the radical (deoxyribose degradation) also found α-lipoic acid to be a hydroxyl radical scavenger. In this study, a rate constant of 4.7×10^{10} $M^{-1}s^{-1}$ was calculated; this is an essentially diffusion-limited reaction rate. Hence, α-lipoic acid appears to be a highly effective scavenger of hydroxyl radical.

There is similar agreement about the ability of α-lipoic acid to scavenge hypochlorous acid. Haenen and Bast[14] and Scott et al.[13] both found that 50 μM α-lipoic acid almost completely abolished the inactivation of α1-antiproteinase by 50 μM HOCl. This is in contrast to glutathione, whose reduced form is a potent scavenger of HOCl, comparable to α-lipoic acid, but whose oxidized form is almost completely ineffective.[14] The authors of this study speculate that the greater reactivity of α-lipoic acid compared to oxidized glutathione may be due to the somewhat strained conformation of the 5-membered ring in the intramolecular disulfide form of α-lipoic acid; there is no such strain on the intermolecular disulfide of glutathione disulfide, perhaps explaining its lack of reactivity in this system.

α-Lipoic acid has been reported to scavenge singlet oxygen in at least four different systems. Two early studies showed that α-lipoic acid reacted with singlet oxygen generated by rubrene autooxidation[15] or by photosensitized oxidation of methylene[16]; these experiments were carried out in organic solvents. Later studies conducted under more physiological conditions also indicate that α-lipoic acid is an effective scavenger of singlet oxygen. Kaiser et al.[17] generated singlet oxygen by thermolysis of endoperoxide and detected it by chemiluminescence; in this system α-lipoic acid reacted with singlet oxygen with a rate constant of 1.38×10^8 $M^{-1}s^{-1}$. In experiments in which singlet oxygen was generated by thermolysis of endoperoxide and detected by single strand DNA breaks, α-lipoic acid was confirmed to be a scavenger of singlet oxygen.[18,19]

Early chemical studies also indicated that α-lipoic acid reacts with hydrogen peroxide.[20] However, in these studies high concentrations of H_2O_2 (30%) and nonphysiological conditions (e.g., one day reaction in acetone) were used. When α-lipoic acid was tested against H_2O_2 in an aqueous environment,[13] no reaction was found, using a peroxidase-based assay system for H_2O_2, for concentrations of α-lipoic acid up to 6 mM.

Two separate studies have failed to show that α-lipoic acid can scavenge superoxide radical. Both used xanthine or hypoxanthine and xanthine oxidase to generate superoxide radical. In one, in which superoxide was detected by ESR using the DMPO spin trap,[12] no reaction was seen (Fig. 2). Similarly, Scott et al.,[13] using cytochrome c reduction to detect superoxide, also found no effect of α-lipoic acid.

The situation regarding the ability of α-lipoic acid to scavenge peroxyl radicals is not so clear-cut. One group[9] reported that α-lipoic acid does not react with peroxyl radicals in either aqueous or lipid environments. Peroxyl radicals were generated using thermolabile azo initiators, either 2,2′ azobis (2-amidinopropane)-dihydrochloride (AAPH) to generate peroxyl radicals in the aqueous phase or 2,2′-azobis (2,4 dimethyvaleronitrile) (AMVN) to generate peroxyl radicals in lipids. Phycoerythrin fluorescence decay was used to detect peroxyl radicals in the aqueous environment, and in lipids (liposomes or rat liver microsomes) they were detected by TBARS or conjugated diene assays. In no case was α-lipoic acid effective in scavenging peroxyl radicals. In contrast, another group,[13] using only an aqueous system and generating peroxyl

Table 1. Antioxidant Effects of α-Lipoic Acid

Oxidant	Scavenged by α-Lipoate?	Test System	Reference
Superoxide radical	No	$O_2^{\cdot-}$ generated by xanthine-xanthine oxidase and detected by ESR using spin traps	12
	No	$O_2^{\cdot-}$ generated by hypoxanthine-xanthine oxidase and detected by $O_2^{\cdot-}$ dependent cytochrome *c* reduction	13
Hydrogen peroxide	No	H_2O_2 added directly and detected by peroxidase-based assay system	13
Hydroxyl radical	Yes	$OH^{\cdot}$ generated by H_2O_2 + $FeSO_4$ and detected by ESR using spin traps or by chemiluminescence using luminol	12
	Yes	$OH^{\cdot}$ generated by H_2O_2 + $FeCl_3$ + ascorbate and detected by deoxyribose degradation. Rate constant for reaction = 4.71×10^{10} M^{-1} s^{-1}	13
Hypochlorous radical	Yes	HOCl added directly and detected by effect on α1-antiproteinase activity	14
	Yes	Same	13
Peroxyl radical	No	Peroxyl radicals generated in aqueous phase by thermal decomposition of 2,2′azobis(2-amidinopropane)-dihydrochloride and detected by fluorescence quenching of phycoerythrin. Peroxyl radicals generated in lipids (liposomes or microsomes) by thermal decomposition of 2,2′-azobis (2,4 dimethylvaleronitrile)	9
	Yes	$CCl_3O_2^{\cdot}$ generated by linear accelerator and detected spectrophotometrically. Rate constant of reaction 1.8×10^8 M^{-1} s^{-1}	13
Singlet oxygen	Yes	$^1O_2$ generated by rubrene autoperoxidation in air-saturated benzene when stimulated at 546 nm and detected by following disappearance of rubrene spectrophotometrically. Rate constant of the reaction = 1×10^8 M^{-1} s^{-1}. DHLA not tested	15
	Yes	$^1O_2$ generated by photosensitized oxidation of methylene in chloroform or methanol; reaction detected by analyzing oxidation products of the methyl ester of LA. DHLA not tested	16
	Yes	$^1O_2$ generated by thermolysis of endoperoxide and detected by infrared chemiluminescence. Rate constant of the reaction = 1.38×10^8 M^{-1} s^{-1}	17
	Yes	$^1O_2$ generated by thermolysis of endoperoxide and detected by single strand DNA breaks	18, 19
Transition metals	Chelator	α-Lipoic acid formed stable complexes with Cu^{2+}, Mn^{2+}, and Zn^{2+} in aqueous solution. Bisnorlipoate and tetranorlipoate formed more stable complexes	23
	Chelator	Same as system to test $^1O_2$. When EDTA was added, the protective effect of LA decreased, suggesting that it was at least partially due to metal chelation	19
	Possible chelator	LA decreased Cd^{2+} toxicity in hepatocytes, but much less effectively than DHLA. The authors speculate that LA is taken up and converted to DHLA, which is the compound exerting the protective effect	21
	No chelation	In rat liver microsomes + $FeSO_4$ + ascorbate, LA had no effect on accumulation of TBARS	22
	Chelator	LA decreased site-specific iron-induced degradation of deoxyribose, suggesting that it chelated the iron	13
	Chelator	Decreased Cu^{2+}-induced oxidation of ascorbate, increased partitioning of Cu^{2+} into octanol, inhibited Cu^{2+}-induced lipid peroxidation	24

radical ($CCl_3O_2^{\cdot}$) by radiolysis of CCl_4 and propan-2-ol, found that α-lipoic acid reacted with this radical with a rate constant of 1.8×10^8 $M^{-1}s^{-1}$; the reaction was followed spectrophotometrically. Although the explanation for the disagreement may lie in the different methodologies of the two groups, further work is necessary to clarify this question.

α-Lipoic acid may also exert an antioxidant effect in biological systems through transition metal chelation. It has been found to reduce Cd^{2+}-induced toxicity in isolated hepatocytes, although the authors speculate that the effect was due to the conversion of α-lipoic acid to DHLA, which was the true chelating agent.[21] Two studies indicate that α-lipoic acid may chelate iron, while one indicates that it does not. Devasagayam et al.,[19] who found α-lipoic acid to be effective against singlet oxygen-induced DNA single strand breaks, found that its effectiveness was lower when EDTA was added to the system, indicating that part of the effect was due to iron chelation. Scott et al.[13] found that α-lipoic acid inhibited the site-specific degradation of deoxyribose by $FeCl_3/H_2O_2$/ascorbate, indicating that it was able remove iron ion bound to deoxyribose. In contrast, in a rat liver microsomal system in which lipid peroxidation was induced by $FeSO_4$,[22] α-lipoic acid did not inhibit peroxidation. Hence, in this area also, further work is needed.

α-Lipoate was found to form stable complexes with Mn^{2+}, Cu^{2+}, Zn^{2+}, with the complex being almost entirely with the carboxylate group.[23] These investigators also examined the complexes formed with bisnorlipoate and tetralipoate, two and four carbon shorter homologues, respectively, of α-lipoic acid. These were found to be more stable than those with α-lipoic acid, presumably because the shorter chain allows the dithiolane ring to also participate in chelation. The effects

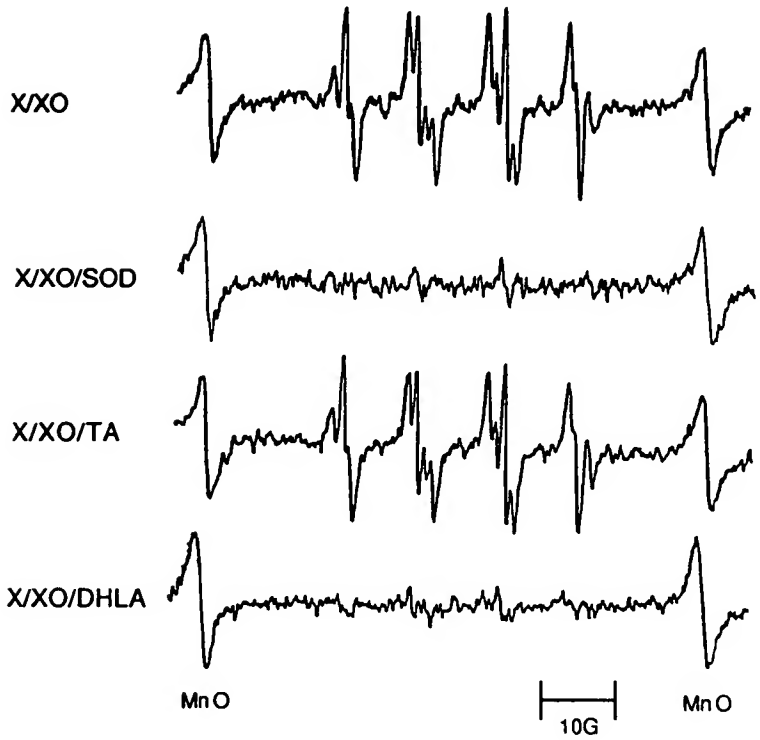

Fig. 2. ESR study of the effects of α-lipoic acid and dihydrolipoic acid on superoxide anion radicals generated by xanthine plus xanthine oxidase. DMPO spin adducts generated by 5 mM xanthine (X) plus 100 μg xanthine oxidase (XO) in the presence of 40 μM DETAPAC in 150 mM KH_2PO_4-KOH (pH 7.4) in total volume of 1 ml [DHPO] = 45 mM; [SOD] = 200 U; [α-lipoic acid (TA) = 5 mM; [dihydrolipoic acid (DHLA) = 5 mM. ESR signals were recorded 3 min after the initiation of xanthine oxidase reaction.

of α-lipoic acid on copper have recently been extended to oxidation situations. α-Lipoic acid was effective in preventing Cu^{2+}-catalyzed ascorbic acid oxidation, increased the partitioning of Cu^{2+} into n-octanol from aqueous solution, and inhibited Cu^{2+}-catalyzed liposomal peroxidation.[24] These observations indicate that α-lipoic acid is a copper chelator.

In summary, α-lipoic acid scavenges hydroxyl radicals, HOCl, and singlet oxygen, but is ineffective against hydrogen peroxide and superoxide radical. It chelates iron, copper, and other transition metals. Further work is required to determine whether α-lipoic acid is effective against peroxyl radicals.

*Dihydrolipoic acid.*Dihydrolipoic acid. With a redox potential of −0.32 V (ref. 25) for the DHLA/α-lipoic acid redox couple, dihydrolipoic acid is a potent reductant; for comparison, the redox potential of the GSH/GSSG couple is −0.24 V (ref. 26)-DHLA will reduce GSSG to GSH, but GSH is incapable of reducing α-lipoic acid to DHLA.[27]

Like α-lipoic acid, DHLA is a potent antioxidant, although there is more uncertainty as to its effects (Table 2). There is agreement that DHLA scavenges hypochlorous acid and peroxyl radicals and probably scavenges hydroxyl radicals. It does not appear to react with hydrogen peroxide or with singlet oxygen. However, there is disagreement among studies as to whether DHLA also is capable of scavenging superoxide radical and whether it acts as an antioxidant or a prooxidant in its interactions with iron ion.

In the systems in which α-lipoic acid was tested for reaction with hypochlorous ion, DHLA was also found to be effective, with about the same scavenging ability.[14]

DHLA is an effective peroxyl radical scavenger in several different systems. Kagan et al.[9] used AAPH or AMVN to generate peroxyl radicals in aqueous or lipid systems, as described for α-lipoic acid, but, unlike α-lipoic acid, DHLA was found to scavenge peroxyl radicals in these systems. It was also found to scavenge peroxyl radicals generated by AMVN in liposomes and detected by fluorescence decay of parinaric acid,[28] and $CCl_3O_2^{\cdot}$ radicals generated in aqueous solution as described for α-lipoic acid[13]; in the latter system a rate constant of $2.7 \times 10^7\ M^{-1}s^{-1}$ was calculated.

DHLA has been shown to have both antioxidant[12] and prooxidant[13] effects in systems in which hydroxyl radical was generated; however, the prooxidant effects, if real, are probably due to DHLA's effects on iron (see below). Both groups used iron salts and hydrogen

Table 2. Antioxidant Effects of Dihydrolipoic Acid

Oxidant	Scavenged by DHLA?	Test System	Reference
Superoxide radical	Yes	$O_2^{\cdot-}$ generated by xanthine-xanthine oxidase and detected by ESR using spin traps. Sulfhydryl content of DHLA decreased during reaction and $[H_2O_2]$ increased. Rate constant of reaction = $3.3 \times 10^5\ M^{-1}\ s^{-1}$	12
	Yes	$O_2^{\cdot-}$ generated by xanthine-xanthine oxidase and detected by epinephrine oxidation. Rate constant of reaction = $7.3 \times 10^5\ M^{-1}\ s^{-1}$	28
	No	$O_2^{\cdot-}$ generated by hypoxanthine-xanthine oxidase and detected by $O_2^{\cdot-}$-dependent nitro-blue tetrazolium reduction.	13
Hydrogen peroxide	No	H_2O_2 added directly and detected by iron-thiocyanate.	29
	No	H_2O_2 added directly. Reaction monitored by measuring thiol content of DHLA	13
Hydroxyl radical	Yes	$OH^{\cdot}$ generated by $H_2O_2 + FeSO_4$ and detected by ESR using spin traps	12
	No (promoted oxidation)	$OH^{\cdot}$ generated by $H_2O_2 + FeCl_3$ + ascorbate and detected by deoxyribose degradation. Prooxidant effect postulated due to reduction of ferrous ion and/or regeneration of ascorbate by DHLA	13
Hypochlorous radical	Yes	HOCl added directly and detected by effect on α1-antiproteinase activity	14
	Yes	Same	13
Peroxyl radical	Yes	Peroxyl radicals generated in aqueous phase by thermal decomposition of 2,2 azobis(2-amidinopropane)-dihydrochloride and detected by fluorescence quenching of phycoerythrin. Peroxyl radicals generated in lipids (liposomes or microsomes) by thermal decomposition of 2,2′-azobis (2,4 dimethylvalcronitrile). Stoichiometry: 1.5 mol peroxyl radicals quenched per mol DHLA	9
	Yes	Same as Kagan et al. 1992. In addition, peroxyl radicals generated in liposomes by AMVN and detected by fluorescence decay of parinaric acid.	28
	Yes	$CCl_3O_2^{\cdot}$ generated by linear accelerator and detected spectrophotometrically. Rate constant of reaction $2.7 \times 10^7\ M^{-1}\ s^{-1}$	13
Singlet oxygen	No	$^1O_2$ generated by thermolysis of endoperoxide and detected by infrared chemiluminescence.	17
	Yes (?)	$^1O_2$ generated by thermolysis of endoperoxide and detected by single strand DNA breaks. The protective effect of DHLA may not be due to direct quenching of $^1O_2$	18, 19
Transition metals	Chelation	DHLA decreased CD^{2+} toxicity in isolated hepatocytes. Also decreased TBARS in Cd^{2+} exposed hepatocytes, indicating that, in the absence of added iron, DHLA's activity is overall antioxidant against peroxidation (contrast to Bast and Haenen, and Scott et al.)	21
	Chelation	DHLA bound iron from ferritin in the ferrous and ferric states.	32, 33
	Prooxidant	In $FeCl_3$-EDTA + H_2O_2 system, accelerated deoxyribose degration Accelerated reaction also when no EDTA in system, suggesting that its iron-binding is not as effective as its iron-reduction However, no prooxidant effect when DHLA was used alone in an Fe (III)-bleomycin-DNA system	13
	Prooxidant	Rat liver microsomes + $FeSO_4 \pm$ ascorbate. DHLA accelerated peroxidation as measured by TBARS	22
	No prooxidant effect	No electron transfer from DHLA to Fe^{3+} as measured by Fe^{2+}-phenanthroline complex. Also, DHLA did not potentiate formation of $OH^{\cdot}$ in Fe SO_4-H_2O_2 system, as measured by ESR (DMPO spin trap)	12
	Some prooxidant effect	In lipid peroxidation in microsomes, induced by AMVN, in the absence of iron (chelated by defreroxaamine), DHLA decreased peroxidation (as measured by TBARS) about 50%. In the presence of iron, DHLA did not decrease peroxidation (although there was no pro-oxidant effect; i.e., the antioxidant and pro-oxidant effects of DHLA balanced in this system). In this system Tetranor-DHLA greatly increased (5X) peroxidation in the presence of iron	28
	Prooxidant	Micromolar DHLA, in the presence of Cu^{2+} ions, caused single-strand nicks in pSP64 plasmid DNA. Other ions were tested in the same system and had no effect: Co^{2+}, Cr^{3+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, Mn^{2+}, and Zn^{2+}. Other thiols also found to cleave DNA in this system in the presence of Cu^{2+}.	34

peroxide to generate hydroxyl radicals. Suzuki et al.[12] detected hydroxyl radicals by ESR, using DMPO to produce spin-adducts and found that DHLA abolished the DMPO-OH signal; Scott et al.[13] detected hydroxyl radicals by deoxyribose degradation and found that addition of DHLA enhanced the process. It is possible that the difference in the results is due to the fact that Scott et al. used ascorbate to reduce ferric iron whereas Suzuki et al. used a ferrous iron salt without ascorbate; DHLA is known to recycle ascorbate (see below) and

thus may have increased ascorbate's effectiveness as a reductant in this system. Scott et al. speculate that this mechanism of direct reduction of ferric iron is the cause of DHLA's prooxidant effect. However, if DHLA were causing the effect through direct reduction of ferric iron, then Suzuki et al. should also have seen a prooxidant effect, and they did not. At any rate, it appears that DHLA scavenges hydroxyl radical, and prooxidant effects may be seen in some systems for producing hydroxyl radical.

In systems similar to those used to test α-lipoic acid for reaction with hydrogen peroxide,[13,29] no reaction was found for DHLA. In addition, Kaiser et al.[17] did not detect scavenging of singlet oxygen by DHLA, using infrared chemiluminescence to detect singlet oxygen. In contrast, DHLA does protect against single strand DNA breaks induced by singlet oxygen (unlike GSH, which increased breaks), but the effect was not necessarily due to quenching of singlet oxygen since there may be several steps in the process at which DHLA could exert an effect.[18,19] Since the more direct method of detecting singlet oxygen, infrared chemiluminescence, did not detect a decrease in its concentration, DHLA probably does not directly scavenge singlet oxygen.

There is major, and as yet unexplained, disagreement on the effects of DHLA on superoxide radical. In a study in which $O_2^{\cdot -}$ was generated by xanthine-xanthine oxidase and detected by ESR using DMPO spin trap,[12] DHLA was found to react with superoxide radical with a rate constant of $3.3 \times 10^5\ M^{-1}s^{-1}$ (Fig. 2). Reaction was confirmed by assaying the sufhydryl content of DHLA, which decreased, and by detecting an increase in H_2O_2, a probable product, in the reaction medium. These results were confirmed by this group in a later study[28] using the same system for generating superoxide radical and competition with epinephrine oxidation to assess DHLA's scavenging ability. In this study a rate constant of $7.3 \times 10^5\ M^{-1}s^{-1}$ was found, in good agreement with the previous work. In contrast to these studies, another group, using a similar superoxide generating system (hypoxanthine-xanthine oxidase) and detecting superoxide by $O_2^{\cdot -}$-dependent nitro-blue tetrazolium reduction, found no scavenging effect of DHLA. It is difficult to explain the discrepancy in the results of the two groups, and further work is clearly indicated.

Perhaps the most crucial question surrounding DHLA is whether it acts as a prooxidant under certain circumstances. There are at least two ways this might happen. First, DHLA may act as a reductant for transition metals, especially iron, and second, DHLA may act to regenerate ascorbate, which is known to reduce iron.

DHLA binds both Fe^{2+} and Fe^{3+}, and possibly may reduce bound Fe^{3+} to Fe^{2+}.[30,31] DHLA also appears to be capable of removing iron from the storage protein ferritin in both the ferrous and ferric states,[32,33] though it does not appear capable of removing iron from heme.[13] If it is indeed capable of reducing iron and if the iron is available for reaction, then its prooxidant effects may be of serious concern in biological systems. Indeed, DHLA was found to act as prooxidant in peroxidation of rat liver microsomes[22] and in production of hydroxyl radicals.[13] In another study in which peroxidation in microsomes was initiated by AMVN,[28] DHLA decreased peroxidation by 50% when desferrioxamine was included in the system but did not decrease peroxidation when desferrioxamine was omitted, indicating that, in the presence of iron, the prooxidant effect of DHLA balanced its antioxidant effect. In this system a homologue of DHLA that is four carbons shorter, tetranor-DHLA, exerted strong prooxidant effects, increasing lipid peroxidation in the presence of iron over five-fold. In addition, DHLA, as well as a number of other thiols and dithiols, has also been shown to induce single-strand breaks in plasmid DNA in the presence of Cu^{2+}, but not in the presence of Fe^{2+} or Fe^{3+}.[34] This is in contrast to the antioxidant Cu^{2+}-chelating effect of α-lipoic acid.[24]

On the other hand, in another system in which iron was used to generate hydroxyl radical, the effect of DHLA was clearly antioxidant,[12] and in this study no electron transfer from DHLA to Fe^{3+}, as measured by formation of Fe^{2+}-phenanthroline complex, was observed. In Cd^{2+} toxicity of isolated hepatocytes, addition of DHLA decreased TBARS,[21] so at least in this system, also, the antioxidant effect of DHLA against lipid peroxidation was greater than any prooxidant effect.

Hence, the question of whether DHLA acts as prooxidant in biological systems is yet to be resolved. DHLA probably increases formation of hydroxyl radical in vitro when ferric ion is included in the system, but the physiological significance of this is unknown. Ascorbate also reduces ferric ion to ferrous ion in vitro; indeed, ascorbate + Fe^{3+} is a standard initiator of lipid peroxidation in in vitro systems, but in vivo under normal conditions, where iron is very tightly bound, this effect of ascorbate is probably unimportant. The same may be true for DHLA, although the possibility that DHLA can remove bound iron from ferritin is cause for concern. Furthermore, the interaction of DHLA with other antioxidants may negate a prooxidant effect in a physiological system. For example, in the study of Bast and Haenen[23] DHLA increased lipid peroxidation induced by ferrous ion, as did ascorbate. However, DHLA exerted an antioxidant effect when included in a system of ascorbate, GSSG, and ferrous

ion, when compared to the same system without DHLA.

These results for α-lipoic acid and DHLA make clear several important areas for future research: (1) The antioxidant actions of α-lipoic acid against peroxyl radical; (2) The action of DHLA against superoxide radical; (3) The effect of DHLA on iron and other transition metals. Furthermore, the uptake and reduction of α-lipoic acid in various tissues must be clarified, since α-lipoic acid and DHLA have different antioxidant effects.

Interactions with other antioxidants

DHLA appears to be able to regenerate other antioxidants, such as ascorbate and (indirectly) vitamin E, from their radical forms. Vitamin E is the major chain-breaking antioxidant that protects membranes from lipid peroxidation.[35] Vitamin E exists in biological membranes in a low molar ratio to unsaturated phospholipids, usually less than 0.1 nmol per mg of membrane protein, or, in other words, one molecule per 1000 to 2000 membrane phospholipid molecules, which are the main target of oxidation in membranes. Lipid peroxyl radicals can be generated in membranes at the rate of 1–5 nmol per mg of membrane protein per minute, yet destructive oxidation of membrane lipids does not normally occur, nor is vitamin E rapidly depleted. Furthermore, deficiency states for vitamin E are remarkably difficult to induce in adult animals. These apparent paradoxes can be explained by "vitamin E recycling," in which the antioxidant ability of vitamin E is continuously restored by other antioxidants. Those antioxidants that recycle vitamin E are vitamin C, ubiquinols, and thiols (Fig. 3).[36,37]

Evidence for vitamin E recycling by DHLA has come from a number of studies. DHLA protects against microsomal lipid peroxidation, but only in the presence of vitamin E[38]; it prolonged the lag phase prior to the onset of low-level chemiluminescence, loss of vitamin E, and the rapid accumulation of thiobarbituric acid reactive substances in normal, but not in vitamin E-deficient, microsomes. α-Lipoic acid was not effective in either system. DHLA could have exerted its effect in this system by directly reducing tocopheroxyl radical or by reducing other antioxidants (such as ascorbate), which then regenerated vitamin E. There may be a weak direct interaction between DHLA and the tocopheroxyl radical; we have found that the presence of DHLA reduces the tocopheroxyl radical ESR signal in liposomes exposed to UV light (unpublished data). The use of UV light, which directly produces tocopheroxyl radicals, eliminates the possiblity of iron chelation or other antioxidant effects of DHLA, and the use of liposomes eliminates the possibility of DHLA acting through other antioxidants. Therefore, in this system it seems likely that DHLA, which partitions mainly in the aqueous phase, is directly reducing tocopheroxyl radicals at the membrane/water interface. However, the effect is weak, and the major recycling of vitamin E by DHLA in biological systems probably occurs through other antioxidants.

Bast and Haenen[39] have proposed that DHLA prevents lipid peroxidation by reducing glutathione, which in turn recycles vitamin E. This proposal is based on the observation that the combination of DHLA and oxidized glutathione, but not DHLA alone, prevented lipid peroxidation induced by Fe^{2+}/ascorbate.[22] Alternatively, Kagan et al.[9] proposed that DHLA protects membranes against oxidation by recycling ascorbate, which in turn recycles vitamin E. Their ESR studies indicated DHLA-mediated reduction of ascorbyl radicals generated in the course of ascorbate oxidation by chromanoxyl (vitamin E or short-chain vitamin E homologues) radicals in DOPC liposomes and also showed that DHLA interacts with NADPH- or NADH-dependent electron transport chains to recycle vitamin E. Ascorbate-dependent recycling of vitamin E by DHLA has also been observed in human low density lipoproteins[40] and erythrocyte membranes.[41] In addition, there is evidence that α-lipoic acid administration in vivo can increase the level of ubiquinol in the face of oxidative stress,[41a] and ubiquinol is known to recycle vitamin E.[41b] Hence, current evidence points to the notion that DHLA can recycle vitamin E via glutathione, vitamin C, ubiquinol, NADPH or NADH, but the relative contributions of each avenue are not well defined.

In fact, protection of other antioxidants by α-lipoic was suggested as early as 1959, by Rosenberg and Culik,[4] who stated, with startling prescience, "α-Lipoic acid, and even more so its dihydro derivative into which it is converted rapidly after entering cellular metabolism, might act as an antioxidant for ascorbic acid and tocopherols." In the studies of Rosenberg and Culik, α-lipoic acid was found to prevent symptoms of both vitamin E and vitamin C deficiency. Recently, we have found similar protective effects of α-lipoic acid administration in tocopherol-deficient hairless mice[42] (Fig. 4). Such results are consistent with a recycling of tocopherol and/or ascorbate by α-lipoic acid, but could also be explained by the ability of α-lipoic acid to spare ascorbate and vitamin E through its separate but overlapping radical-scavenging effects.

α-Lipoic acid also causes an increase in intracellular glutathione. Busse et al.[43] added α-lipoic acid to murine neuroblastoma and melanoma cell lines, and observed a dose-dependent increase in GSH content of 30–70% compared to untreated controls. These investigators found similar elevations in lung, liver, and

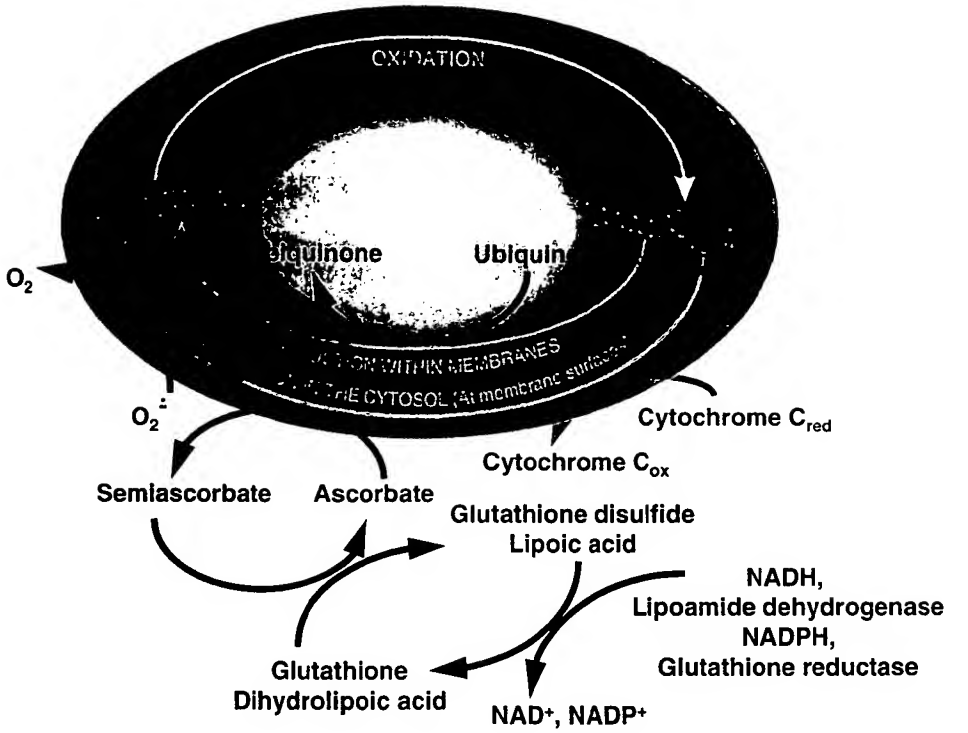

Fig. 3. Vitamin E recycling. The tocopheroxyl radical, formed during oxidation of α-tocopherol (shown in the membrane), may be reduced back to α-tocopherol by a number of compounds, including ubiquinol, cytochrome c, and ascorbate. Ascorbate can be regenerated through reaction with thiols such as glutathione or lipoic acid. These can be returned to their reduced forms by various mechanisms, drawing on the reducing power of NADH or NADPH. Adapted from Scientific American.

kidney cells of mice injected (IP) daily with doses of 4, 8, or 16 mg/kg α-lipoic acid for 11 days. These results have been confirmed in human Jurkat cell lines, in which intracellular concentrations of GSH increase approximately 50% in 5 h after addition of α-lipoic acid to the culture medium (D. Han, G. Handelmann, personal communication). Such elevations in GSH cannot be explained by reduction of GSSG, since GSSG is normally present at less than 10% the concentration of GSH.[44] These intriguing observations are yet to be explained.

Thus, it appears that α-lipoic acid and DHLA act as antioxidants not only directly, through radical quenching and metal chelation, but indirectly as well, through recycling of other antioxidants and possibly though induction of increased intracellular levels of glutathione.

Redox regulation of proteins and influence on protein folding

Thiolation of proteins has been reported to be a protective mechanism against oxidative stress, as well as affecting the function of some thiol-containing proteins.[44a] Glutathione is the most abundant thiol in mammalian cells[44] and may be a primary agent involved in redox regulation of protein thiols under normal conditions. α-Lipoic acid and DHLA do not appear to be present in the unbound state under normal physiological conditions, but after dietary supplementation both forms appear in various tissues in the unbound form.[42] DHLA also has a lower redox potential than GSH, as well as being of lower molecular weight. These factors lead to the possibility that administration of exogenous α-lipoic acid may influence intracellular function not only through antioxidant actions but also through affecting the redox status of thiol-containing proteins, such as throredoxin, enzymes, and transport proteins. Dihydrolipoate and dihydrolipoamide can reduce thioredoxin,[47,48] a small ubiquitous protein that functions to transfer electrons in various biochemical processes.[49] Spector et al.[50] proposed that thioredoxin may be a physiological electron acceptor from lipoamide. Glucose transport has also been shown to be enhanced by DHLA in a number of systems,[76–78] and it is thought that this stimulation may be due to reduction of sulfhydryl groups involved in the regulation of insulin-stimulated glucose transport.[81]

DHLA has also been found to influence physiologically important proteins through mechanisms other than disulfide reduction. DHLA reduces metmyoglobin and ferrylmyoglobin to oxymyoglobin.[45] DHLA also

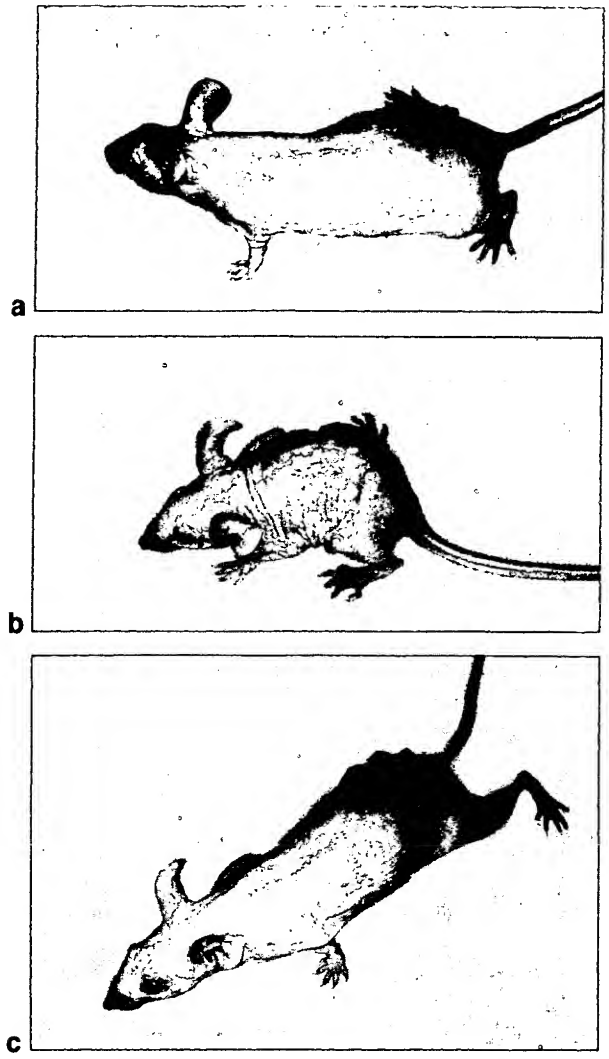

Fig. 4 Adult 12-week old hairless mice after 6 weeks of (a) normal control diet, (b) vitamin E deficient diet, and (c) vitamin E deficient, α-lipoate-supplemented diet. The animal on the vitamin E deficient diet shows symptoms of vitamin E deficiency with muscular dystrophy and weight loss.

elicits glandular kallikrein-induced prolactin proteolysis by acting upon prolactin to refold the molecule into conformations that are glandular kallikrein substrates.[46] Hence, DHLA may exert an influence over intracellular metabolism by means other than antioxidant effects, but the physiological significance of this remains to be elucidated.

Effects on gene expression

There has recently been a great deal of interest in the effects of oxidants and antioxidants on signal transduction and gene expression in both normal and abnormal conditions. In this regard α-lipoic acid and DHLA have been investigated in terms of their effect on the transcription factors NF-κB,[51] which regulates the expression of genes such as human immunodeficiency virus type 1 and those involved in inflammatory responses.[52] The effects of α-lipoic acid and DHLA on the expression of c-fos have also been studied.

NF-κB is regulated through redox mechanisms,[53,54] and sulfhydryl groups such as Cys62 in the p50 subunit have been identified as important in this regulation.[55] There are two steps in the process of NF-κB activation and action that may be influenced by thiol antioxidant such as α-lipoic acid. Early steps involve the activation of NF-κB and dissociation from an inhibitory subunit, IκB. These are apparently at least partly under redox control, with oxidation stimulating activation and dissociation. The binding of activated NF-κB to DNA involves cysteine residues whose redox status is also important, with reduced cysteine apparently enhancing binding. Hence, the effects of thiol-containing antioxidants can be complex. For example, overexpression of thioredoxin inhibits TPA-induced activation of NF-κB,[55a] whereas in an in vitro system thioredoxin enhanced binding of NF-κB to DNA.[55,55b] Similarly, incubation of human Jurkat T-cells in medium containing 4 mM α-lipoic acid completely inhibits NF-κB activation induced by tumor necrosis factor or phorbol myristate 13-acetate.[56] Recently, the DNA binding activity of NF-κB was found to be enhanced by DHLA and inhibited by α-lipoic acid. Inhibition of NF-κB DNA binding induced by a nonreducing environment or by exposure to a thiol-oxidizing agent, diamide, was reversed by DHLA.[57] The interplay of these compounds and their effects on NF-κB in a physiological cellular environment is difficult to predict.

The effects of both α-lipoic acid and DHLA on the expression of the growth-regulating gene c-fos have also been investigated.[58] Jurkat T cells preincubated with either α-lipoic acid or DHLA were exposed to TPA, an activator of c-fos expression. Cells incubated in DHLA exhibited less c-fos mRNA expression, compared to controls, whereas those preincubated with α-lipoic acid exhibited greater expression compared to controls. Active oxygen species are produced by cultured cells stimulated by TPA,[59–61] and superoxide production is enhanced in TPA-stimulated leukocytes.[62] The suppression of c-fos expression by DHLA but not by α-lipoic acid may be due to the fact that DHLA scavenges superoxide, whose production may be involved in enhancing c-fos expression, whereas α-lipoic acid does not.

Apoptosis is another process in which intracellular oxidation is thought to play a role. In rat thymocytes exposed to methylprednisolone or etoposide, inducers of apoptosis, preincubation with DHLA or lipoamide inhibited apoptosis. Lipoic acid had no effect (S. Orrenius personal communication).

Although it appears that DHLA and/or α-lipoic acid may influence gene expression at one or more levels, the exact mechanisms and significance have yet to be elucidated. This area holds great promise.

EXPERIMENTAL AND CLINICAL THERAPEUTIC STUDIES

α-Lipoic acid administration has been shown to be effective in preventing pathology in various experimental models in which reactive oxygen species have been implicated. Before considering these studies, however, it is necessary to discuss whether α-lipoic acid is absorbed as a dietary supplement, to what degree it is taken up by tissues, whether it is reduced to DHLA, and whether it is metabolized to shorter chain homologues.

Absorption, uptake, intracellular reduction, and metabolism of α-lipoic acid

There is no doubt that administration of dietary α-lipoic acid has an effect on whole-body physiology. The work of Rosenberg and Culik[4] made this clear because α-lipoic acid, supplemented in the diet of rats and guinea pigs, protected against the symptoms of vitamin E or vitamin C deficiency. In later experiments,[63] animals were fed a diet supplemented with α-lipoic acid for 12 days, then liver, skin, and brain homogenates were tested for susceptibility to lipid peroxidation. The amount of peroxidation induced by AMVN (measured as TBARS after 40 min of incubation with AMVN) decreased 79% in liver, 64% in skin, and 50% in brain. These experiments indicate that α-lipoic acid in the diet has an antioxidant effect, both at the tissue level and at the level of the entire animal, but they do not prove that α-lipoic acid is, in fact, even absorbed intact.

Experiments using radiolabeled α-lipoic acid indicate that the compound is absorbed. When 14C-labeled lipoic acid was administered to rats, either as an IP injection or orally, via stomach tube, 80% of the administered radioactivity was either excreted or found in the tissues.[6] This was true even in animals whose intestinal flora had been killed, so it is unlikely that the animals were absorbing metabolites produced by bacteria in the gut.

The next question is how much of the α-lipoic acid is converted to DHLA intracellularly.

α-Lipoic acid administered to a variety of cell and tissue systems appears in the medium as DHLA.[65] However, the intracellular fate was unknown. In a more recent study,[10] lipoic acid was added to the culture medium for human fibroblasts or Jurkat T-cells, at concentrations from 1 to 4 mM. The concentrations of both α-lipoic acid and DHLA in the cells and in the culture medium were determined by HPLC with electrochemical detection at various times up to 2 h. The intracellular concentration of DHLA in the Jurkat cells reached 1.5 mM within 10 min. It was also found that the cells released DHLA into the medium.

The results indicate that normal mammalian cells are capable of taking up α-lipoic acid, reducing it to DHLA, and releasing DHLA. Hence, the effects of both α-lipoic acid and DHLA may be present both intracellularly and extracellularly when α-lipoic acid alone is administered extracellularly. This has important implications in, for example, the use of α-lipoic acid supplementation to prevent LDL oxidation.

Finally, a recent study, which repeated and extended the experiments of Rosenberg and Culik,[4] involving vitamin E-deficient rats, confirmed that α-lipoic acid is absorbed and is converted to DHLA in tissues.[42] The hairless mice were used as a model for studying the effects of E deficiency with or without α-lipoic acid supplementation, because hairless mice display obvious symptoms of deficiency within 5 weeks (Fig. 4). Mice were fed either a normal diet, a vitamin E-deficient diet, or an E-deficient diet supplemented with 1.65 g α-lipoic acid/kg diet . α-Lipoic acid supplementation completely prevented symptoms of vitamin E deficiency. Both α-lipoic acid and DHLA (unbound) were measured after 5 weeks on the various diets in liver, kidney, heart, and skin. Only the mice fed the diet supplemented with α-lipoic acid displayed any unbound α-lipoic acid or DHLA in these tissues. Total α-lipoic acid was highest in heart (3.42 ± 2.20 nmol/g wet weight) and lowest in liver (0.60 ± 0.33 nmol/g). In all tissues DHLA was detected as well as α-lipoic acid, and represented from 21% to 45% of the total. Metabolites of α-lipoic acid were not measured.

This is the strongest evidence to date that α-lipoic acid, administered in the diet, accumulates in tissues, and that a substantial portion of the α-lipoic acid is converted to DHLA. However, the absorption and reduction of α-lipoic acid in humans is not as well-studied; endogenous plasma levels of α-lipoic acid were 1–25 ng/ml and of DHLA 33–145 ng/ml in 6 healthy volunteers,[66] but plasma levels of α-lipoic acid and DHLA in individuals supplemented with α-lipoic acid remains to be thoroughly investigated.

Another area that requires further study is the extent to which exogenously supplied α-lipoic acid is metabolized to shorter chain homologues, which may have different effects than α-lipoic acid itself. In studies of administration of radiolabeled α-lipoic acid to rats,[67] much of the excreted α-lipoic acid was in altered forms, including the β-oxidation products bisnorlipoate, tetranorlipoate, and beta-hydroxybisnorlipioc acid. It is not known what proportion of administered

α-lipoic acid is converted to these metabolites; in one study of α-lipoic acid administered to cultured T-lymphocytes, no formation of shorter chain-length beta-oxidation products of lipoic acid was observed over 2 h.[10] Hence, the question of how much α-lipoic acid is converted to its shorter chain homologues is still unanswered. However, it is worth considering their effects.

Reduced forms of bisnorlipoic acid and tetranorlipoic acid react with superoxide and are more reactive than dihydrolipoate. Bimolecular kinetic rate constants were $17.7 \times 10^5\ M^{-1} \cdot s^{-1}$ and $10.9 \times 10^5\ M^{-1} \cdot s^{-1}$ for the superoxide reaction for bisnorlipoate and tetranorlipoate, respectively, compared to $7.3 \times 10^5\ M^{-1} \cdot s^{-1}$ for DHLA.[28] Reduced forms of bisnorlipoate and tetranorlipoate were also found to scavenge peroxyl radicals both in aqueous solution and in liposomes.[28]

Reduced forms of bisnorlipoate and tetranorlipoate also have marked prooxidant effects. In the absence of iron chelator, reduced forms of bisnorlipoate and tetranorlipoate potentiated hepatic microsomal lipid peroxidation induced by AMVN-generated peroxyl radicals.[28] The reduced form of tetranorlipoate was extremely potent in inducing lipid peroxidation.

In summary, α-lipoic acid is absorbed from the diet, transported to the tissues, and taken up by cells where a large proportion is rapidly converted to DHLA. Hence the effects of both α-lipoic acid and DHLA must be considered when proposing mechanisms for therapeutic effects. The extent to which α-lipoic acid and DHLA are metabolized, and the exact antioxidant/prooxidant balance of their metabolites, is yet to be established. These metabolites may also play a significant role in the observed effects of treatment with α-lipoic acid.

Diabetes

α-Lipoic acid has potential applications for many aspects of the pathology of diabetes. In Type I (insulin-dependent) diabetes, destruction of pancreatic β-cells causes loss of insulin secretion, whereas in Type II (noninsulin-dependent) diabetes insulin resistance of peripheral tissues is the major problem. α-Lipoic acid has potential preventive or ameliorative effects in both Type I and Type II diabetes.

Many of the complications induced by diabetes, including polyneuropathy and cataract formation, appear to be mediated by oxygen free radical generation[68]; diabetic patients have elevated serum levels of thiobarbituric acid reactive substances compared to nondiabetics.[69] Since α-lipoic acid and DHLA have been shown to prevent diabetes-induced biological alterations,[70–73] the relationship between the role of oxidative stress in diabetes-induced complications and the antioxidant properties of α-lipoic acid and dihydrolipoic acid is of particular interest. Glycation of proteins may also play a role in these and other pathologies accompanying the disease. α-Lipoic acid has been shown to have promise for preventing glycation reactions.

Effects in animal models of type i diabetes. Type I diabetes results from β-cell destruction by immunological or inflammatory attack. One animal model for this type of diabetes is the nonobese diabetic mouse. When diabetes development was accelerated in nonobese diabetic mice by cyclophosphamide administration, 60% of the mice developed diabetes within 1–3 weeks.[74] If α-lipoic acid was given (10 mg/kg, IP) for 10 days before and 10 days after cyclophosphamide administration, only 30% of the mice developed diabetes. This effect could be due to suppression of nitric oxide release by macrophages, which has been shown to occur in the presence of DHLA,[75] as well as to scavenging of other reactive oxygen species released by inflammatory cells which attack the pancreatic β-cells. The large suppression of diabetic induction by α-lipoic acid should prompt further research in this area.

Effects on glucose uptake in type ii diabetes. One of the major features in the pathogenesis of Type II diabetes is insulin resistance. Most Type II diabetics are hyperinsulinemic, hence no insulin therapy is warranted. Improvement of insulin action on glucose transport activity in skeletal muscle has therefore been the basis for a number of studies seeking to find therapeutic interventions that will be successful. As skeletal muscle tissue is the major sink in the body for glucose following a meal, agents that enhance glucose uptake by skeletal muscle are potentially useful in the long-term treatment of Type II diabetes.

α-Lipoic acid enhances glucose utilization in isolated rat diaphragm,[76] heart,[77] and cultured myotubes.[78] In the latter study, the authors report that the naturally occurring R-form stereoisomer of lipoic acid is more effective than the S-form in stimulating glucose uptake. R-α-lipoic acid-stimulated glucose transport is associated with the translocation from the cytosol of GLUT-1 and GLUT-4 glucose transporters to the plasma membrane; this has an additive effect on insulin stimulated glucose transport. S-α-Lipoic acid stimulates transport to a lesser extent, but does not promote translocation and actually inhibits insulin action on glucose uptake.

Using the obese Zucker rat as an animal model of obesity in insulin resistance, Henriksen and colleagues[79] demonstrated that α-lipoic acid treatment markedly increased the uptake of glucose [as 2-deoxyglucose] in the absence or presence of insulin. Acute treatment with α-lipoic acid as a single dose of 100

mg/kg body weight for 1 h, or chronic treatment (30 mg/kg for 10 days) resulted in improved insulin-stimulated 2-DG uptake in epitrochlearis muscles by over 50% in both cases.

Jacob and colleagues[80] have also demonstrated in humans with Type II Diabetes that α-lipoic acid administration (1000 mg given intravenously) enhanced insulin-stimulated whole body glucose disposal by about 50%. These results correlate well with the findings of Bashan et al.[78] and Henriksen et al.[79] in the myotube and rat epitrochlearis muscle studies reported above.

A biochemical explanation for these findings is required. It has been suggested that the exogenously supplied α-lipoic acid may bring endogenous levels of α-lipoic acid, known to be low in diabetic animals[70] back to normal. Alternatively, α-lipoic acid may react with cellular sulfhydryl groups, believed to be involved in the regulation of insulin-stimulated glucose transport.[81] The effect may also be due to LA's antioxidant function. At present it is not possible to distinguish among these possibilities. Although the mode of action of lipoic acid stimulation of glucose disposal remains to be elucidated, these studies are consistent with work in animal and cell model systems, and suggest the need for a larger scale clinical study.

Glycation reactions and atherosclerosis. Glycation of proteins may be an underlying factor in a number of the pathologies of diabetes,[82] and free radicals may be involved in the process.[68] Although several mechanisms have been postulated for the pathogenesis of chronic diabetic complications, protein glycation and oxidation by glucose (glycoxidation) are plausible working hypotheses.[83,84,68]

α-Lipoic acid has been tested in a number of model systems, particularly serum albumin and lysozyme, where the inhibition of enzyme activity or the extent of glycosylation has been found to be protected against by incubating in glucose together with α-lipoic acid.[85] In another study, Kawabata and Packer[86] found that noncovalent hydrophobic binding of α-lipoic acid to albumin was involved in its protective effects (Fig. 5). Also in this in vitro test system, both the R and S enantiomer forms of α-lipoic acid behaved similarly. In addition, both α-lipoic acid and DHLA protected albumin from glycation in the same manner, demonstrating that the preventive effect is independent of its redox state. Since α-lipoate has a hydrophobic carbon chain, it is likely that α-lipoate binds to albumin by hydrophobic interactions similar to fatty acids. By comparing α-lipoate with a shorter chain homologue, tetranor-lipoate, it was shown that α-lipoate is much more protective against glycation. In fact, tetranor-lipoate did not show any protective effect, demonstrating the importance of the hydrophobic interactions between albumin and α-lipoate in protection against glycation.

Glycated human serum albumin binds fewer fatty acids than does nonglycated albumin,[87] suggesting that glycation sites are adjacent to fatty acid binding sites. In an early step of albumin glycation, α-lipoic acid may protect BSA from glycation by masking the glycation site through hydrophobic binding. Although covalent interaction with serum albumin has been reported to inhibit the protein glycation, noncovalent interaction of Diclofenac (Voltaren) with human serum albumin was also reported to inhibit glucose attachment to human serum albumin.[88] α-Lipoic acid may act in a similar manner.

Protection of human low density lipoprotein [LDL] against glycation by α-lipoate was also investigated. If α-lipoate inhibits LDL glycation, this could have clinical significance because diabetic patients have a higher frequency of atherosclerosis than nondiabetic individuals.[89] LDL is a large particle of more than 1.5 million kD with a cholesterol: phospholipid surface, a core composed primarily of cholesterol ester, and a single protein (apo B100), which is interdigitated in the surface. Physically, apo B100 has both hydrophilic and hydrophobic domains; the hydrophobic portion of apo B in LDL is submerged in the lipid structure, and in this respect it differs from albumin. α-Lipoic acid was found not to protect LDL from glycation in vitro[86] (Fig. 5). These findings also emphasize the significance of the hydrophobicity of α-lipoate in protection against glycation. Schepkin et al.[90] also reported using NMR techniques that α-lipoate binds to albumin, but not to LDL.

However, although α-lipoic acid was not found to protect LDL in the short term, the long-term effects of glycation involve further reaction to Amadori products, and eventually the formation of advanced glycosolated end-products (AGE). These steps may involve oxidative mechanisms. It is worth noting in this regard that α-lipoic acid has been found to be protective of human LDL exposed to oxidative stress[40,91] (Fig. 6). Hence, over the long-term, α-lipoic acid may help protect diabetics from the atherosclerosis that is a common component of the disease.

Diabetic polyneuropathy. Endoneural blood flow and oxygen tension are reduced in experimental diabetic neuropathy. This process of endoneural hypoxia is associated with an increase of oxidative stress and an impairment of the nerve conduction velocity.[92]

Antioxidants such as glutathione have been shown to prevent neuropathy in animal models,[93] and α-lipoic acid induces sprouting of neurites in culture.[94]

Clinical trials have been carried out on the effects

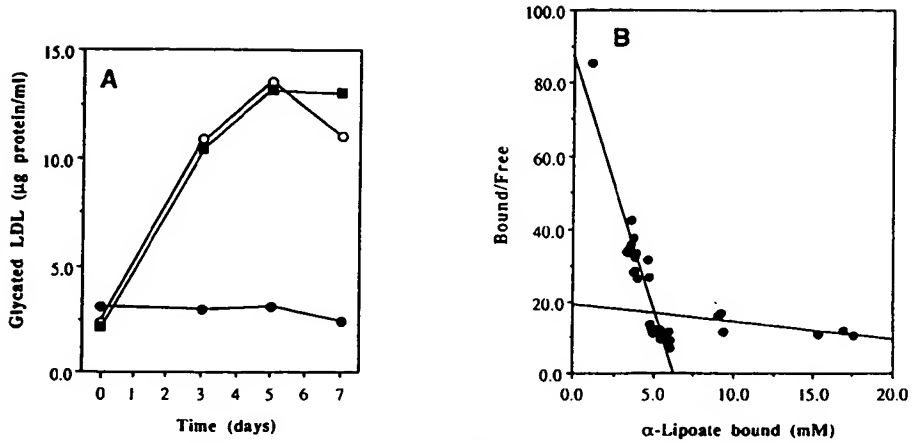

Fig. 5. (A) Effect of α-lipoate on LDL glycation. LDL (1 mg protein/ml) was incubated with 200 mM glucose in the presence (open circles) or absence (filled squares) of α-lipoate (20 mM) in Hepes-saline (pH 7.4) at 37°C. At the indicated times, glycated LDL were separated by affinity chromatography and protein concentrations measured. Values are averages of duplicate observations. LDL was also incubated without glucose as a negative control (filled circles). (B) The binding of α-lipoate to BSA. BSA (1 mM) was incubated with various concentrations of α-lipoate in Hepes-saline (pH 7.4) at 37°C for 18 h. The sample was centrifuged in a centricon 30 and the α-lipoate concentration in the filtrate was measured by absorption spectroscopy (ϵ = 150 $M^{-1} \cdot cm^{-1}$).

of α-lipoic acid supplementation on diabetic neuropathy, and its use for this condition is approved in Germany.

In one placebo-controlled double blind study,[95] 21 days of intravenous administration of α-lipoic acid (200 mg daily) caused alleviation of some clinical symptoms: before treatment six patients had moderate pain and four had severe pain, and after treatment five patients had no pain, four had moderate pain, and only one had severe pain. Despite these dramatic clinical improvements, neurophysiological measures, such as vibration sense and nerve conduction velocity, showed no change with IV lipoic acid administration. The authors note that 21 days may have been too short a time in which to observe changes in neuronal characteristics.

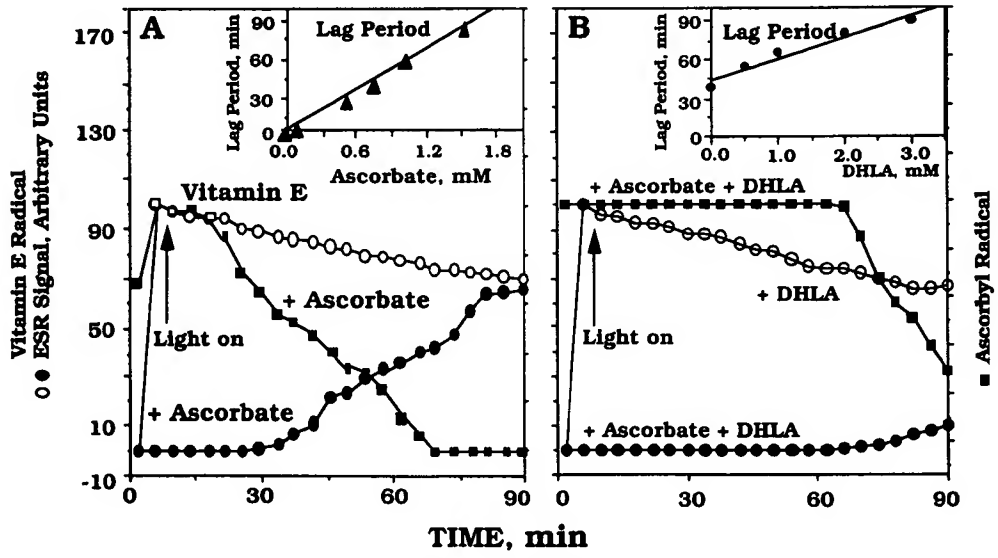

Fig. 6. Time course of UV-induced vitamin E chromanoxyl and ascorbyl ESR signals in LDL suspensions. (A) Effect of ascorbate: ascorbate, 500 μM. Insert: dependence of the lag period (during which the vitamin E chromanoxyl radical ESR signal was not observed) on the concentration of ascorbate. (B) Effect of dihydrolipoic acid plus ascorbate, ascorbate 500 μM; DHLA 2 mM. Insert: dependence of the lag period (during which vitamin E chromanoxyl radical ESR signal was not observed) on the concentration of adihydrolipoic acid (the ascorbate concentration was 500 μM). Incubation medium (100 μl) contained: LDL samples with endogenous vitamin E (6.2 nmol/mg protein, 14 mg protein/ml), ascorbate, 500 μM inphosphate buffer, pH 7.4, at 25°C. All values are given as a percentage of the maximum magnitude obtained. Vitamin E chromanoxyl radicals were generated by irradiation with UV light (290–400 nm).

In a longer study, α-lipoic was compared to vitamin B1 as treatment for diabetic neuropathy in a single-blind study. α-Lipoic acid (600 mg/day) or vitamin B1 (400 mg/day) were administered intravenously and intramuscularly, respectively, to diabetics for 3 weeks, followed by 12 weeks of oral administration of the same dose.[96] Pain and parathesia were reduced in the α-lipoic acid patients when compared with the vitamin B1 patients, but again in this study no improvement in motor or sensory nerve conduction velocity could be demonstrated due to lipoic acid administration. However, these authors point out that a period of months or years is required to observe improvement in diabetic neuropathy due to normalization of blood glucose levels, so it is not surprising that no measurable neurophysiological changes occurred over a period of 15 weeks of α-lipoic acid supplementation. However, clinical improvement, evaluated by blinded observers, has clearly occurred in these studies.

In a nonblinded study in which several parameters were studied,[97] diabetic patients (both type I and type II) were given oral supplements of either α-lipoic acid (600 mg/day for 2 weeks, then 300 mg/day for 10 weeks), α-tocopherol (1575 IU daily), or selenium (100 μg daily), and compared to controls (no supplements, no placebo) after 12 weeks. Blood malondialdehyde decreased from 14.4 μmol/l to 10.9 μmol/l in the α-lipoic acid group. Similar reductions were seen in the α-tocopherol and selenium groups. No change was seen in the control group. Albuminuria decreased 50%, 46%, and 26% in the α-lipoic acid, α-tocopherol supplemented, and selenium supplemented groups, respectively. There was clinical improvement in neurological symptoms in all supplemented groups but not in the control group. In addition, while retinopathy worsened in 5 of 9 in the control group, there was only one case of worsening of retinopathy in any of the antioxidant supplemented groups. This study showed that mitigating oxidation, whether by α-lipoic acid supplementation or supplementation with other antioxidants, improved biochemical and functional parameters in diabetics; however, the study suffered from lack of placebo control and lack of double-blind.

Cataracts. Another common complication of diabetes is cataract formation. α-Lipoic acid dietary supplementation has been shown to prevent cataract formation caused by BSO-induced inhibition of GSH synthesis in newborn rats, while sparing lens ascorbate, tocopherol, and GSH.[98] 100% of BSO-treated rats developed cataracts, but if they also received α-lipoic acid, the incidence of cataract formation dropped to 40%. Significant protection of lens ascorbate, tocopherol, and glutathione was also observed in the lipoate-supplemented rats compared to nonsupplemented.

Also, in vitro diabetic cataractogenesis in rat lens cell cultures exposed to high concentrations of glucose was prevented by α-lipoic acid[99] (Fig. 7). Our group[100] has hypothesized that α-lipoic acid prevents oxidative stress in diabetic conditions by sparing vitamin C, whose transport is affected by the disease. Since vitamin C and glucose share the same carrier in noninsulin dependent tissues, the elevated blood glucose in diabetes competitively inhibits the cell entry of vitamin C, thus resulting in localized intracellular vitamin C deficiency (Fig. 8). Exogenously supplemented α-lipoic acid utilizes other transport systems to enter the cells, is converted to dihydrolipoate, and recycles vitamin C. This would explain its protective effect in diabetic cataractogenesis, as well as other complications of diabetes.

Conclusions. α-Lipoic acid has been shown to have a number of beneficial effects, both in prevention and treatment of diabetes. α-Lipoic acid may act in a number of ways that are especially protective in diabetes. It prevents β-cell destruction leading to Type I diabetes. It enhances glucose uptake in Type II diabetes. It prevents glycation reactions in some proteins. Its antioxidant effects may be particularly useful in slowing the development of diabetic neuropathy and cataractogenesis, and this may be especially significant in alleviating diabetes-induced reduction in intracellular vitamin C levels.

Very few of these effects will manifest as objective improvement over the course of weeks or even months. This is demonstrated in trials for treatment of neuropathy, which lasted up to 12 weeks, in which objective improvement was not observed but clear subjective improvement was present, even in double-blinded studies. Given the array of beneficial effects of α-lipoic acid that have been shown in both animal and human diabetes, large, long-term, well-controlled studies seem warranted. It is unrealistic to expect dramatic effects in weeks, since diabetic complications develop over years and decades.

Ischemia–reperfusion injury

Ischemia–reperfusion injury occurs when a burst of free radicals is produced during reoxygenation of tissue that has become hypoxic. It is important in cardiac tissue (especially with the introduction of clot-dissolving drugs for the treatment of heart attack) and in brain. Agents that prevent ischemia–reperfusion injury may therefore prove important in the treatment of cardiac infarct, during open-heart surgery, and in the treatment of stroke and other conditions that cause interruption of blood flow to the brain.

In vitro, DHLA prevents ischemia–reperfusion in-

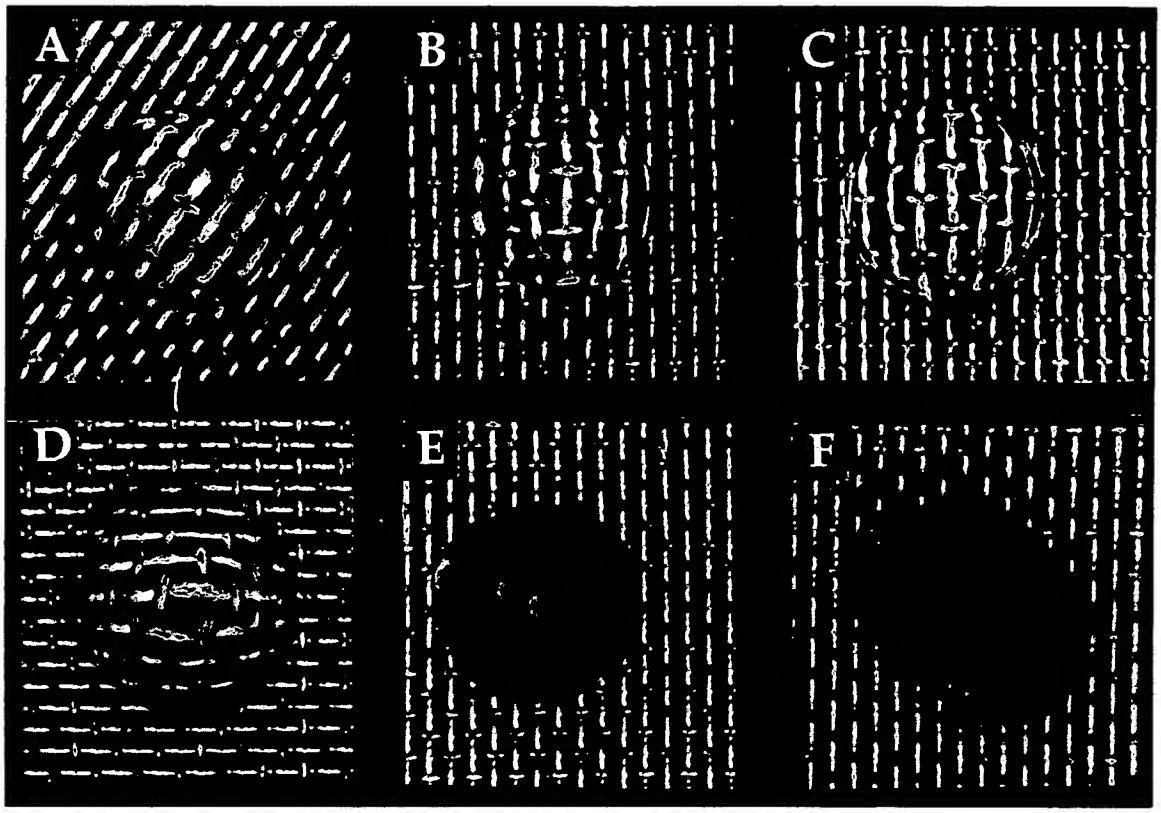

Fig. 7. Effect of α-lipoate on glucose induced lens opacity. Lenses as seen through a dissection microscope, after 8 days of incubation. Rat lenses with intact capsules were incubated in Modified Eagle Medium 199, with (A) normal glucose (5.56 mM) and 1 mM R-α-lipoic acid, (B) normal glucose (5.56 mM) and 1 mM S-α-lipoic acid, (C) normal glucose (5.56 mM) and 1 mM racemic α-lipoic acid, (D) elevated glucose (55.6 mM) and 1 mM R-α-lipoic acid, (E) elevated glucose (55.6 mM) and 1 mM S-α-lipoic acid, and (F) elevated glucose (55.6 mM) and 1 mM racemic α-lipoic acid.

duced changes in fluidity and polarity of rat heart mitochondria.[101] Several studies extend these experiments to more closely match in vivo situations.

Serbinova et al.[102] reported that hearts from rats fed α-lipoic acid were protected against ischemia–reperfusion injury induced in an isolated perfused Langendorff heart system. α-Lipoic acid preserved vitamin E in heart tissue, improved postischemic left ventricular functional recovery, and decreased lipid peroxidation and lactate dehydrogenase leakage (a marker of membrane damage). Haramaki et al.,[103] using Langendorff and working heart systems, further observed that the protective effects of dihydrolipoic acid against rat myocardial ischemia–reperfusion injury are dependent on vitamin E, suggesting that α-lipoic acid functions in this system by regenerating tocopherol from the tocopheroxyl radical. Assadnazari et al.,[104] using a working heart system in an NMR magnet, found that DHLA added to the reperfusion buffer accelerated the recovery of aortic flow during reperfusion. DHLA also appeared to increase ATP synthesis in the heart.

The brain is another area where ischemia–reperfusion injury can have serious consequences; for example, in head trauma, subarachnoid hemorrhage, stroke, or cardiac arrest. Prehn et al., in a study with middle cerebral artery occlusion in mice, demonstrated that in this model of focal ischemia, treatment with DHLA, but not α-lipoic acid, reduced the size of the infarct.[105,106] The authors also noted a hypoglycemic effect at the doses used (100 mg/kg body weight). In another model of rat brain ischemia–reperfusion injury, in which the carotid arteries are occluded, pretreatment with α-lipoic acid decreased mortality from 80% to 20%; this marked decrease in mortality was accompanied by protection of glutathione (which was depleted in controls) and decreased levels of lipid peroxidation product formation.

In other ischemia–reperfusion systems, Boveris et al.[107] reported that α-lipoic acid administration prevented rat intestinal short-term ischemia–reperfusion induced overshoot of chemiluminescence, which is indicative of an increased steady-state level of singlet oxygen and an increased rate of lipid peroxidation. Boveris also observed that α-lipoic acid can inhibit xanthine oxidase activity; xanthine oxidase is postulated to play an important role in the mechanism of ischemia–reperfusion injury.

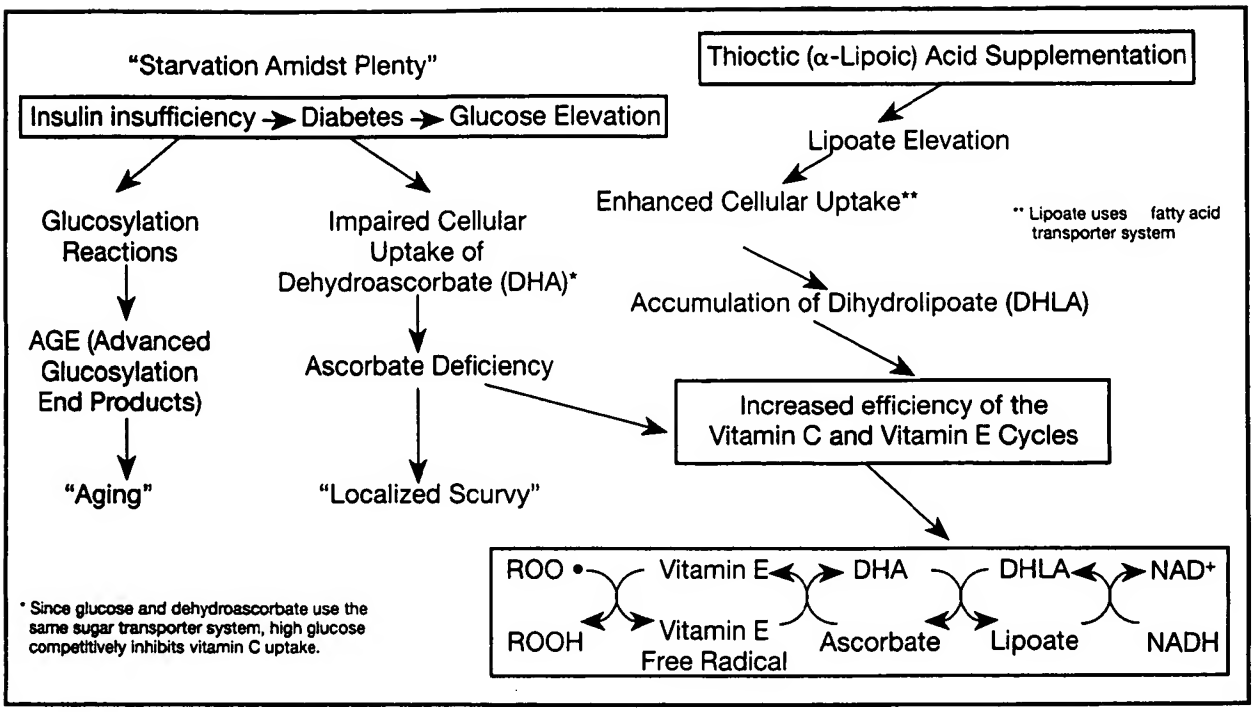

Fig. 8. Schematic diagram of the pathobiochemical situation in diabetes mellitus and the possible correction by oral administration of α-lipoic acid. Since glucose competitively inhibits the absorption of ascorbic acid into the cells, α-lipoic acid increases the efficiency of the vitamin C cycle and thereby also activates the vitamin E cycle.

Thus, in a number of ischemia–reperfusion model systems, DHLA/α-lipoic acid has been shown to be effective in ameliorating or preventing damage. In none of these studies were the cellular contents of DHLA or α-lipoic acid measured, so it is difficult to say with certainty exactly what the mechanism of protection was. In the cardiac system the effect appears due to recycling.

Liver diseases

α-Lipoic acid is often used for therapy in conditions that involve liver pathology. The basis for such treatment is the metabolic role of α-lipoic acid, not its antioxidant properties. The two most extensively studied conditions are mushroom poisoning and alcoholic liver degeneration. There is little evidence that α-lipoic acid is useful in either of these conditions.

Mushroom poisoning. There have been many case studies in which treatment with α-lipoic acid has been associated with complete recovery from mushroom (Amanita) poisoning.[108,109] However, 10–50% of victims recover without α-lipoic acid treatment, so such reports may be coincidental. In animal studies, α-lipoic acid has proved completely ineffective as a treatment for Amanita poisoning, and some groups recommend its elimination from a treatment regimen for this condition.[110]

Alcoholic liver disease. Several studies indicated that α-lipoic acid administration might be beneficial in liver disease, especially alcoholic liver disease.[111–113] However, these studies suffered one or more of the following flaws: lack of control groups, lack of statistical analysis, or the use of other treatments in addition to α-lipoic acid.

In a controlled, double-blind, long-term study, α-lipoic acid had no effect on the course of alcohol-related liver disease.[114] Twenty patients with precirrhotic liver disease received α-lipoic acid (300 mg/daily, orally) for 6 months, and 20 patients received placebo. Twenty-two of the patients abstained from alcohol, and the other 18 reduced daily alcohol intake. Significant improvements in blood parameters (serum aspartate transaminase, serum gamma glutamyl transpeptidase, and mean corpuscular volume) occurred in the abstainers, as well as histological improvement in 17; 5 of the reduced intake group also showed histological improvement but there was no significant improvement in blood characteristics. α-Lipoic acid did not influence the course of the disease.

Nf-κb activation, hiv, and aids

Oxidative stress may play a role in several aspects of HIV infection, including activation of virus replica-

tion,[115] immunosuppression,[116,117] and tumor initiation and promotion.[118] HIV-infected patients have been reported to be deficient in various antioxidants.[119-121] Hence, there may be a role for antioxidant therapy in retarding the progress of the disease.

Following this reasoning, a pilot study was recently conducted on the effects of α-lipoic acid supplementation on blood antioxidant status and blood peroxidation products in HIV-positive individuals.[122] Twelve patients (HIV positive, classified CDC IV) received oral doses of 150 mg of lipoate three times daily for a period of 14 days. At the end of that time, 9 of 10 patients exhibited increases in plasma ascorbate, 7 of 7 showed increased plasma glutathione, 8 of 9 had decreased plasma malondialdehyde, and 7 of 9 had decreased 4-hydroxynonenal. Accompanying these changes, the number of T-helper cells increased in 6 of 9 patients, and the T-helper/T-suppresser cell ratio improved in 6 of 10 patients.

Also, in cultured cells, α-lipoic acid and dihydrolipoic acid prevented HIV replication,[123] the activation of NF-κB transcription factor,[56] which are regulated by oxidative stress.

These studies suggest that further research is warranted on the effect of α-lipoic acid in HIV infection.

Neurodegenerative diseases

Tissues of the central nervous system may be especially vulnerable to oxidative stress because of their constant high rate of oxygen consumption and high mitochondrial density. Mitochondria inevitably produce free radicals as "byproducts" of normal oxidative metabolism,[124,125] and these free radicals damage the mitochondrial DNA.[126] The defective proteins coded for by the damaged DNA can lead to synthesis of mitochondria in which components of the electron transport chain preceding the damaged protein become reduced, leading to greater free radical production[127-129] and more mitochondrial damage, in a vicious cycle. Such a vicious cycle may be responsible, in part for neurodegenerative diseases.

Support for this view comes from the observation of high degrees of oxidative damage as well as damaged mitochondria in tissues from patients with neurodegenerative disease.[129-134] Logical therapy or prevention would therefore involve antioxidant treatment.

α-Lipoic acid is a good candidate as an antioxidant agent in neurodegenerative diseases. It can interrupt the chain at several points: by competing for free transition metals as a chelator,[19,21] by scavenging hydroxyl or superoxide radicals,[12] and by scavenging peroxyl radicals.[9] Few other antioxidants possess this kind of versatility, and it will be interesting to see what effect α-lipoic acid has in various neurodegenerative disorders.

Studies are underway in animals. For example, a recent study examined the effect of α-lipoic acid on memory loss in aging mice.[135] Many species, including man, monkeys, rats, and mice, exhibit aging-related cognitive deficits that may be caused, at least in part, by oxidative stress. In mice, α-lipoic acid (100 mg/kg body weight for 15 days) improved performance in an open-field memory test—in fact, the α-lipoic acid-treated animals performed better than young animals 24 h after the first test (though the difference was not significant).[135] Treatment with α-lipoic acid did not improve memory in young animals. The α-lipoic acid-treated animals exhibited decreased age-related *N*-methyl-D-aspartate (NMDA) receptor deficits (B_{max}) compared to controls, but showed no improvement in muscarinic, benzodiazepine, or a_2-adrenergic receptor deficits. The authors concluded that α-lipoic acid's free radical scavenging ability may improve NMDA receptor density, leading to improved memory. These intriguing results suggest further tests in other species, including humans.

Recently, Greenamyre et al.[136] observed that the intraperitoneal administration of α-lipoic acid or DHLA reduced the rat striatum lesions induced by excitotoxins which affect NMDA receptors, which may lead to calcium influx and generation of nitric oxide and other free radicals.[137] Both α-lipoic acid and DHLA treatment decreased the area of lesion by 50%.

Another way in which mitochondria may be important in neurodegeneration is through alterations in their effects on calcium homeostasis. In this regard, the recent report of Christof Richter (personal communication) that α-lipoic acid inhibits mitochondrial calcium transport may be relevant to its beneficial effects noted in neurodegenerative disorders noted by Greenamyre.[136]

Ischemia–reperfusion injury, induced by head trauma, subarachnoid hemorrhage, stroke, or cardiac arrest, is another potential source of free radical damage to neuronal structures. The protective effect of α-lipoic acid in this condition has already been discussed (see Ischemia–Reperfusion Injury). In addition, α-lipoic acid treatment of rats exposed to inhalation of n-hexane (constant exposure to 700 ppm) delayed the onset of severe neuropathy by 3 weeks: control rats displayed severe motor neuropathy by 6 weeks, while in rats which also received α-lipoic acid, severe neuropathy did not appear until the ninth week.[138]

Radiation injury

Irradiation is known to produce a cascade of free radicals, and antioxidant compounds have long been

used to treat irradiation injury. α-Lipoic acid, but not DHLA, protected against radiation injury to hematopoietic tissues in mice, and increased the LD_{50} from 8.67 to 10.93 Gy.[139] α-Lipoic acid administration to murine neroblastoma cells[43] increased cell survival after irradiation from 2% to about 10%, and the effect correlated with an increase of the intracellular GSH/GSSG ratio induced by α-lipoate. These results extended to mice irradiated with 8 Gy; α-lipoic acid administration (16 mg/kg) increased survival rates from 35% in untreated animals to 90% in α-lipoic acid-treated animals.

The worst recent widespread human radiation exposure occurred after the Chernobyl accident. People continue to live in areas where the soil is contaminated with 15–40 Ci/km2, and are thus exposed to constant, low-level radiation. In an effort to determine whether antioxidant treatment might help prevent damage due to such constant exposure, Korkina, Afanas'ef, and Diplock recently examined the effects of 28 days of antioxidant treatment on a variety of blood and urinary parameter in children living in areas affected by the Chernobyl accident.[140] Using spontaneous chemiluminescence as a marker for blood peroxidation, they found that treatment with α-lipoic acid alone lowered this value to that seen in nonradiation-exposed children; α-lipoic acid + vitamin E treatment further lowered chemiluminescence to below-normal values; vitamin E alone was without effect. Urinary excretion of radioactive metabolites was also lowered by α-lipoic acid but not by E, presumably due to chelation by α-lipoic acid. Liver and kidney functions were also normalized by α-lipoic acid treatment.

Hence it appears that α-lipoic acid, alone or together with vitamin E, is an effective treatment for radiation exposure, lessening indices of oxidative damage and normalizing organ function.

Cigarette smoke effects on plasma proteins

Although the exact mechanisms by which cigarette smoke (CS) exposure causes such pathologies as atherosclerosis, pulmonary emphysema, and cancer have not yet been elucidated, it is known that CS contains a number of free radical species.[141,142] Since many of the diseases caused by smoking involve, at least in part, free-radical-mediated processes, it has been proposed that the free radicals in CS contribute to these diseases. The effects of CS on lung lining fluids has been investigated using plasma as a model system. Freshly obtained human plasma is exposed to "puffs" of gas-phase or whole CS, and antioxidants and markers of oxidative damage are measured. In this system, ascorbic acid is consumed first, followed by protein thiol groups.[143,144] Lipid hydroperoxides as well as protein carbonyls (a marker of protein oxidation) appear.[143,145] Exogenously supplied DHLA is protective against CS-induced oxidation of antioxidants, proteins, and lipids.[144] DHLA also partially protected polymorphonuclear leukocytes from the damaging effects of CS.[146] This effect may be due to scavenging of oxidants in the aqueous or lipid phases, or to regeneration of ascorbic acid that has been converted to ascorbyl radical as it is oxidized by CS components. In this regard, DHLA could play a role in minimizing the pathologic consequences of smoking. To extend these studies to the in vivo situation, it will be necessary to know what levels of DHLA are achieved in plasma after dietary supplementation with α-lipoic acid.

Heavy metal poisoning

The possible chelating effects of α-lipoic acid, together with its antioxidant effects, make it a good candidate for the treatment of heavy metal poisoning. For arsenite, cadmium, and mercury, especially, it may be effective. Grunert[147] demonstrated that α-lipoic acid administration completely protected mice and dogs from arsenite poisoning, if the ratio of α-lipoic acid to arsenite was at least 8:1. This was true even if the α-lipoic acid was administered after severe symptoms of poisoning were already seen. Similar complete protection was seen for Hg^{2+} poisoning in mice, although at low doses (2:1 molar ratio) α-lipoic acid appeared to potentiate the lethal effect of Hg^{2+}. α-Lipoic acid was ineffective against lead and gold poisoning in mice in this study. Exposure of isolated hepatocytes to α-lipoic acid or DHLA resulted in the amelioration of cadmium^{2+}-induced membrane damage, lipid peroxidation, and the depletion of cellular glutathione.[21] These findings were extended to a rat model, in which α-lipoic acid at a dose of 30 mg completely prevented cadmium-induced lipid peroxidation in brain, heart, and testes.[148] In this study it also completely abolished cadmium-induced decreases in the activities of Ca^{2+}-, Na^{+}-, and Mg^{+}-ATPases in these organs. α-Lipoic acid administration to rats also increased biliary excretion of injected Hg^{2+} (the major route of excretion) by 12–37-fold,[149] but dramatically decreased biliary excretion of Cd^{2+}, methyl mercury, Zn^{2+}, and Cu^{2+}. It is not clear whether this was due to chelation of these metals in the circulation by α-lipoic acid or other effects of α-lipoic acid. α-lipoic acid administration has also been found to greatly increase the rate of elimination of radiomercury in rabbits.[150] Hence, α-lipoic acid may be of clinical value in the treatment of mercury and cadmium poisoning. Its effect on other heavy metals is not yet completely clear.

Chagas disease

Chagas disease is common in South America and is caused by the protozoan *Trypanosoma cruzi*. It is treated with benznidazole, but side effects in up to 50% of cases are a major problem. In a double blind study of 50 patients with Chagas disease,[151] 25 were treated with benznidazole + α-lipoic acid (20 mg/day) and 25 with benznidazole + placebo. In the benznidazole + α-lipoic acid group, only 8% of the patients developed side effects and all of the patients were able to complete treatment, whereas in the benznidazole + placebo group 44% of the patients developed side effects and two had to abandon treatment. α-Lipoic acid is a beneficial adjuvant therapy in the treatment of this disease.

Side effects

Neither animal nor human studies to date have shown serious side effects with administration of α-lipoic acid.[152] The LD_{50} is approximately 400–500 mg/kg following intravenous administration in rats and 400–500 mg/kg after oral dosing in dogs. In long-term oral supplementation at doses sufficient to reduce body weight gain, no functional or laboratory adverse effects were seen in animals. No evidence suggests carcinogenic or teratogenic effects, but it is recommended that pregnant women avoid taking supplemental α-lipoic acid until more data are available.[152] Although few adverse effects are noted in studies of administration of α-lipoic acid, it has been observed in thiamine deficient rats that a high dose of lipoic acid (20 mg/kg), delivered intraperitoneally, caused fatal complications; thiamine-sufficient rats suffered no adverse effects from α-lipoic acid supplementation, and the action of lipoic acid in the deficient rats could be prevented by administration of thiamine just prior to lipoic acid administration.[153] The animals in this study were severely thiamine-deficient, showing frank polyneuritis from thiamine deficiency before lipoic acid was administered, and the dose of lipoic acid was large; nevertheless, it may be prudent that any group likely to be severely thiamine-deficient, for example, alcoholics, should receive supplemental thiamine if α-lipoic acid is given.

In humans, side effects include allergic skin reactions and possible hypoglycemia in diabetic patients as a consequence of improved glucose utilization with high doses of α-lipoic acid.[152]

CONCLUSIONS

α-Lipoic acid and its reduced form, DHLA, have been referred to as "a universal antioxidant" that functions in both membrane and aqueous phases.[9] Both α-lipoic acid and DHLA have substantial antioxidant properties. These include their ability to directly quench a variety of reactive oxygen species, inhibit reactive oxygen-generators, and spare other antioxidants.

A number of experimental as well as clinical studies point to the usefulness of α-lipoic acid as a therapeutic agent for such diverse disease conditions as myocardial and cerebral ischemia-reperfusion injury, heavy-metal poisoning, radiation damage, diabetes, neurodegenerative diseases, and AIDS. High doses of α-lipoic acid are approved in Germany for treatment of diabetic polyneuropathy. Furthermore, the interesting antioxidant properties of α-lipoic acid and its interaction with other important antioxidants like vitamin E, ascorbate, and glutathione will provide a fertile field for continued research.

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EXHIBIT C

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Free Radical Processes in Plant Tissue Cultures: Implications for Plant Biotechnology Programmes

By

E.E. BENSON[1], W.J. MAGILL[1] & D.H. BREMNER[1]

Key words: Oxidative stress, free radicals, lipid peroxidation, tissue cultures, antioxidants, morphogenesis, totipotency.

Summary

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Tissue culture techniques have an important role in the genetic improvement of crop plant species. However, genomic instability, in vitro recalcitrance, and loss of morphogenetic potential are limiting factors affecting plant biotechnology initiatives. The objectives of this review are to evaluate the experimental evidence for the occurrence of free radical processes in plant tissue cultures. Relationships between oxidative status and in vitro plant development will be assessed and the implications that these factors may have for crop plant biotechnology explored. Achieving a better understanding of free radical mechanisms in tissue cultures may have useful applications in crop plant improvement.

Introduction

Tissue culture has a key role in the biotechnological improvement of crop species and the totipotent property of plants (the ability to regenerate whole plants from single cells) is widely exploited. Thus, in vitro manipulations underpin crop improvement initiatives involving genetic manipulations, reproductive technologies, and plant conservation. Tissue culture techniques are also involved in phytosanitary regulation and international germplasm exchange. However, there exist three major limitations to the use of in vitro methods: (1) certain species are unresponsive (recalcitrant) to in vitro manipulation; (2) cultures maintained in the

[1] School of Molecular and Life Sciences, University of Abertay Dundee, Bell St. Dundee, DD1 1HG, Scotland.

dedifferentiated state for extended periods, loose their totipotent capacity and (3) there is an increased propensity for genetic instability in plants maintained in vitro. These factors can severely restrict biotechnology programmes which are dependent upon the maintenance of genetic fidelity and whole plant regeneration. Whilst attempts have been made to elucidate the basis for the deleterious changes generated during in vitro manipulation, these largely describe symptoms rather than underlying causes. It is now widely accepted that free radical processes in animal systems are associated with development as well as cellular and genetic degeneration. Studies of free radical mechanisms in in vitro plant cultures may thus have important implications for plant biotechnology programmes.

Assessments of free radical processes in tissue cultures include direct EPR detection, and measurements of secondary oxidation products and antioxidant status. Table 1 summarises evidence for the involvement of free radical processes in culture responses using these different approaches. Thus, free radical species have been directly detected in *S. tuberosum* using EPR spectroscopy. Preliminary evaluations of EPR spectra suggests that semiquinone and carbon-centred peroxy radicals are formed during cellular dedifferentiation (BAILEY & al 1994).

Several procedures have been used to assess secondary oxidation product formation and malondialdehyde, thiobarbituric acid reactive substances (TBARS), hydroxyalkenals, conjugated dienes, lipid peroxides and fluorescent oxidation products have been detected in a wide range of plant cultures derived from very diverse species (Table 1). Assessments of antioxidant enzymes and sulfhydryl group (SH) status indicate that the formation of secondary oxidation products can be related to dynamic changes in antioxidant capacity. Whilst studies of free radical processes in in vitro plant systems are limited, our own findings confirm those of others (e.g. BIEDINGER & SCHNABEL 1991, CUTLER & al. 1989). Currently, there now exists considerable evidence to support the view that free radicals are a component of in vitro plant development. Interestingly, the application of exogenous antioxidants to plant cultures is used to stimulate morphogenetic responses and ameliorate deleterious oxidative stress (JOY & al. 1988, ISHII 1988).

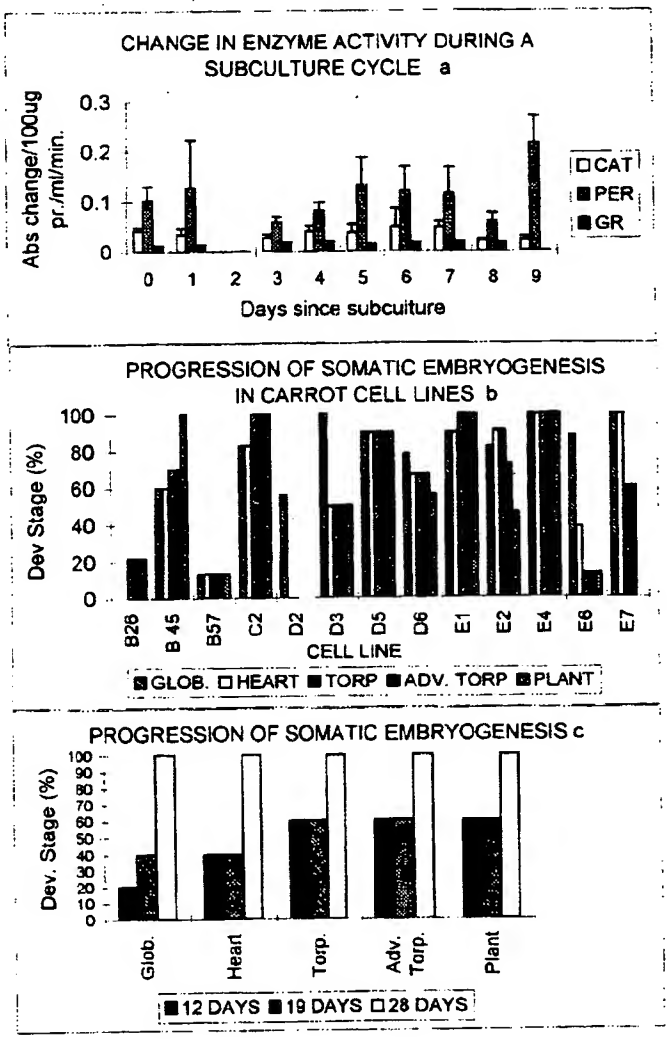
Do free radical processes have a role in vitro plant development?

Our own studies (Table 1) show well-defined trends concerning the pro- and antioxidant status of in vitro plant cultures. There is an overall enhancement in oxidative activity at the onset of a developmental transition. Thus, callus induction in *V. vinifera* stem explants was accompanied by a most pronounced increase in TBARS and aqueous soluble fluorescent oxidation products. Changes in SH status, and catalase activity also occur, though superoxide dismutase (SOD) levels were less influenced by developmental change. Following active dedifferentiation the "peak" in pro- and antioxidant activity declined in the proliferating callus, to levels just above or similar to the original explant (BENSON & ROUBELAKIS-ANGELAKIS

1992, 1994). Similar responses were observed in dedifferentiating *S. tuberosum* explants, where callus induction accompanied a transitory increase in free radical activity (BAILEY & al. 1994). Somatic embryogenesis is proposed as the system of choice for studying in vitro development (ZIMMERMAN 1993). Thus, a differentiated stem explant dedifferentiates on auxin containing callus induction medium. Under these conditions the cells can only proliferate and differentiation is suppressed, however, on transfer to hormone-free medium competent cells undergo somatic embryogenesis Studies involving regenerative pathways demonstrate the same trend. Thus, the hydroxyalkenal content of *D. carota* callus cultures increased on transfer to embryo induction medium and a similar, although less defined increase in malondialdehyde was observed (ROBERTSON & al. 1995).

Recently, we have characterised the morphogenetic characteristics of 13 different embryogenic callus cultures of *D. carota* (Fig. 1). These will be used for the study of free radical processes in in vitro ageing. Preliminary findings show that catalase, glutathione reductase and peroxidase activities can vary within a standard culture cycle and they may provide useful markers of in vitro development (Fig. 1a). Maintenance of embryogenic cultures for prolonged periods in the dedifferentiated state can cause a between different clones. The embryogenic pathway is highly complex (Fig. 1c) and future investigations will use these 13 morphogenetically characterised lines (Fig. 1b) to assess, in more detail, the relationships between culture morphogenesis, culture age and oxidative enzyme status. Ascertaining the direct role of antioxidants and free radicals in in vitro ageing and development is likely to be difficult. Free radical activity is associated with stress and it is a contributory factor in culture recalcitrance (Table 1). Clearly, many products of secondary oxidative stress are cytotoxic (ESTERBAUER & al. 1988) and their production and interaction with macromolecules could actually promote genotoxicity and enzymatic dysfunction in culture systems. However, sole consideration of the negative aspects of free radical activity risks over simplification. Oxidative processes may also have a direct role in in vitro development. Morphogenesis is a dynamic process controlled by the application of exogenous, potent, plant growth regulators. These have the capacity to alter primary oxidative metabolism and directly influence hormonal transduction pathways involving activated oxygen species (VAN EMMERIK & al. 1992, GUNSE & ELSTNER 1992). Indeed, the lipid peroxidation product, jasmonic acid can stimulate in vitro plant morphogenesis (RAVNIKAR & GOGALA 1990). Similarly, EARNSHAW & JOHNSON 1985 correlated glutathione status with morphogenetic competence in carrot suspension cultures. GSH levels were higher in proliferating cultures compared to differentiating cultures, and they concluded that development occurs in a more oxidising environment. It is thus essential to consider both positive and negative aspects of in vitro oxidative metabolism in plants.

Recently, HAGEGE 1996 discussed two hypotheses concerning oxidative processes in plant cell culture habituation. Habituation is the condition in which cell cultures spontaneously achieve hormonal autonomy and proliferate in culture



without the need for exogenous plant growth regulators. HAGEGE discusses the "permanent stress" hypothesis proposed by ARBILLOT & al. 1991 and LE DILLY & al. 1993 that states that low levels of catalase and peroxidase in habituated cultures result in the accumulation of activated oxygen species and free radicals. These promote the generation of lipid peroxides that impair cell development. A second "antioxidant adaptive strategy" hypothesis, is favoured by HAGEGE who proposes that cells challenged with a stressful culture environment develop a new "adaptive"

cell population in which free radical scavenging ability is enhanced. This adaptation reduces the capacity of the metabolic pathways that normally generate activated oxygen species and promotes increased free radical scavenging properties in habituated cells. As metabolic and developmental pathways have a dependency on activated oxygen (for example, those involving lipoxygenase) this may explain the frequently observed inability of habituated cultures to differentiate (HAGEGE 1996). These hypotheses give useful "working" models for directing future studies of free radical processes in plant cultures. However, habituated cultures would not be a system of choice in applied biotechnology programmes for which the maintenance of totipotency and genetic stability is the primary concern. For these applications it is more important to determine the role of free radical mechanisms in maintaining genetic fidelity and regenerative capacity. For example, evaluations of non-habituated cell cultures of *O. sativa* provide evidence that loss in embryogenic capacity is associated with enhanced lipid peroxidation, the accumulation of secondary lipid peroxidation products and a decrease in catalase and peroxidase activities (BENSON & al 1992).

Free radical processes in plant cultures: Implications for plant biotechnology

At present there exists no direct evidence to implicate free radicals, activated oxygen species and/or their reaction products as causal agents in either genetic or epigenetic instability in plant cultures. Free radicals are known to mediate genotoxic changes in animal cells (ESTERBAUER & al. 1988) and it may be prudent to explore this possibility in plants. Changes in the pro- and

Fig. 1. Profiles of catalase, peroxidase and glutathione reductase activities and morphogenetic development in *D.carota* callus cultures

LEGEND: (a) Changes in catalase (CAT) peroxidase (PER) and Glutathione (GR) activities (on the basis of protein, (pr)) measured during a sub-culture cycle of callus originally derived from hypocotyl explants of *D. carota* cultivar Early Nantes (culture age = 15 months). Evaluations were performed on replicates of 3-9 callus extracts and error terms are standard deviations (data is not available for time = 2 days). Catalase and peroxidase assays were performed as described previously (BENSON & al. 1992), glutathione reductase was measured using the method of GOLDBERG & SPOONER 1983. (b) Variation in embryogenic capabilities of 13 different clonal callus cultures of *D.carota*: cultures B26, D3, E6 are from cultivar (cv) Autumn King, cultures B45 and B57 are from cv Golden King, cultures C2 D2, E1,2 and 4 are from cv Early Nantes, D6 and E7 are from cv Chanteney Red Core and D5 from cv Saint Valery. Culture ages comprise 15 months (E1, E2, E4, E6, E7); 19 months (B45, B26, B57); 20 months (D2,D3,D5 D6) and 32 months (C2). Morphogenetic evaluations (as % colonies exhibiting developmental progressions) were performed 28 days after transfer to hormone free embryo-induction medium. A total of 10 different callus colonies were evaluated for each cv (c) Progression of somatic embryogenesis of culture E4 (Early Nantes) evaluated during a 28 day embryo-induction cycle following transfer to hormone free medium. Progression in embryo development is characterised: as globular and heart morphology (early stages) torpedo [torp] (mid stage) and advanced [adv.] torpedo and plant (late stage). Carrot cultures were maintained and manipulated as described by ROBERTSON & al. 1995 and ZIMMERMAN 1993.

antioxidant status of plant cultures, particularly those which are dedifferentiated may be related to totipotent capacity (Table 1, Fig. 1). Manipulation of the oxidative status of plant cultures may provide a useful means of maintaining morphogenetic competence or overcoming recalcitrance and this may best be explored in relation to the application of exogenous hormones and cell signalling pathways (GUNSE & ELSTNER 1992).

Table 1.Summary of experimental evidence implicating the involvement of free radical and antioxidant mechanisms in plant culture responses.

Species/culture system	Evidence	Reference
Helianthus annuus protoplasts	generation of ethane negatively correlated with protoplast division	BIEDINGER & SCHNABEL 1991
Cereal protoplasts	accumulation of peroxidation products and a decrease in antioxidants correlated with protoplast recalcitrance	CUTLER & al. 1989
Vitis vinifera callogenesis	catalase, SH, TBARS and fluorescent peroxidation products increased at onset of dedifferentiation, SOD decreased	BENSON & ROUBELAKIS-ANGELAKIS 1994
Vitis vinifera micropropagation	fluorescent peroxidation products increased during micropropagation, SOD activity did not change	BENSON & ROUBELAKIS-ANGELAKIS 1992
Oryza sativa cell competence	lipid peroxidation higher in cell lines which had lost or were losing embryogenic competence, differences in SOD activity between lines not observed, peroxidase and catalase activity higher in competent cells	BENSON & al.1992
Solanum tuberosum callus induction	EPR spectroscopy signals assigned to enhanced free radical activity at onset of callogenesis	BAILEY & al. 1994
Daucus carota callus proliferation, somatic embryo induction	Detection of TBARS, malondialdehyde, hydroxyalkenals in proliferating callus, enhancement of HNE levels during early somatic embryo induction	ROBERTSON & al. 1995
Beta vulgaris habituated cells	Hypothesis for in vitro habituation proposed: cells undergo a hyperantioxidant scavenging which reduces pro-oxidant activity	HAGEGE 1996

Future studies and cautionary points

Substantial correlative evidence supports the premise that free radical mechanisms have a role in in vitro plant development. Future studies must utilise stringent experimental strategies and employ more discerning methods for the analysis of free radical products. Meaningful investigations can only be carried out on well characterised cultures and parallel analyses of pro- and antioxidants must be performed. Oxidative status and morphogenetic capacity may be interrelated and both may be influenced by subculture cycle, culture age and genotype (Fig.1). We strongly caution the use of tissue cultures as "models" for the study of stress

responses without prior knowledge of the culture's developmental history. Habituation, culture age and competence greatly influence the oxidative status of cultures and may confound experimental interpretations. Finally, the application of transformation technologies offers immense potential for the study of antioxidant systems in plants (BADIANI & al. 1996). However, if transgenic in vitro systems are to be utilised it may be useful to be aware of the interactions between culture competence, age and oxidative status.

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X. RELATED PROCEEDINGS APPENDIX

None